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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,290,204**, on November 22, 1999, by **THE UNIVERSITY OF BRITISH COLUMBIA,** assignee of Steven Pelech, for "Kinase Network Modelling".

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KINASE NETWORK MODELLING

Background of the Invention

The human genome is believed to contain about 120,000 genes, which are present in each of the 50 trillion nucleated cells of the body. At any given moment in each cell, however, only about 20% of these genes are actively transcribed for the production of the proteins that they encode. The unique complement of proteins that each cell expresses is referred to as its "proteome". It is critical that the levels of expression and activity of the proteins in a cell are tightly regulated. This is achieved through a subset of about 10% of these proteins, which are dedicated to cell communications and control One of the largest classes of proteins involved in cell signaling are enzymes called protein kinases. Protein kinases control other proteins by catalyzing their phosphorylation, which is a process that can be reversed by protein phosphatases. Often protein kinases operate within signalling pathways that are further integrated into networks. The unique complement of protein kinases expressed in a cell is referred to herein as its "kineome".

There are different amino acids that can be phosphorylated by protein kinases. Most commonly, this occurs on serine and threonine, and to a much lesser extent on tyrosine. For example, in skin cells called fibroblasts, 90% of measurable protein kinases catalyze the serine and threonine phosphorylation of proteins, whereas a different class of related enzymes generally carry out tyrosine phosphorylation. Therefore, protein-serine/threonine kinases are responsible for most protein phosphorylation events in cells. For convenience, these kinases are referred to herein as protein-serine kinases, although they also phosphorylate proteins on threonine.

Approximately fifty of the hundred or so known genes that have been directly linked to induction of cancer (i.e. oncogenes) encode protein kinases. The remainder of the oncogenes specify proteins that either activate kinases or are phosphorylated by kinases. Most of the oncogene-encoded protein kinases are tyrosine-specific, but several are protein-serine kinases such as protein kinase C, Raf1, Akt, ILK-1, Tpl2, and Mos. Although the findings are less direct, aberrant cell signalling through protein kinases has also been linked to cardiovascular disease, diabetes, inflammation, arthritis and other immune disorders, and neurological disorders such as Alzheimer's disease. Over 400 human diseases have been linked to defective signalling through protein kinases.

Essentially all signalling proteins, if they are not already protein kinases, appear to be regulators of protein kinases or their substrates. As signal transduction networks govern and co-ordinate all cellular functions, including cell structure, metabolism, reproduction, adaptation, differentiation and death, knowledge of the structure of signalling networks will permit a complete understanding of how the cell operates under a diversity of conditions. Kineome analysis represents a key step in this process.

Kineome analysis will yield many practical benefits. The presence and state of activity of diverse protein kinases and their pathways are indicators of how a cell perceives its internal and external environments and how it is responding. Therefore, by monitoring the kineome, it will be feasible to obtain a molecular diagnosis of a disease condition. Moreover, by inhibiting or activating the appropriate protein kinases by pharmacological intervention, antisense or gene therapy, it would be possible to "reprogram" the kineome to better treat the disease condition. For example, in cancer, the gain of function of one of over fifty different oncogene-encoded protein kinases may be pivotal for neoplastic transformation of cells. Inhibition of the appropriate kinase or its downstream effectors could block the improper proliferative signalling and initiate apototic processes leading to programmed death of the tumor cells.

The genome sequencing projects for man, mouse and other organisms will permit the rapid identification of all of the protein kinases within the next few years. Elucidation of the connections between these protein kinases in different cells will allow kineome analysis to then reach its full potential. Over two thousand different protein kinases are thought to be encoded by the human genome and several hundred are likely to be expressed within any given cell. All of these protein kinases will have to be tracked for a complete elucidation of the architecture of kinase networks. This is feasible through the employment of kinase specific probes.

Most eukaryotic protein kinases are evolutionarily related, i.e. the genes of almost all protein-serine/threonine and protein-tyrosine kinases display sequence identity. In particular, there are 16 amino acid residues located in 10 subdomains in the catalytic region of protein kinases that are highly conserved. These amino acids allow unambiguous identification of novel protein kinases following analysis of the primary structures of proteins as revealed by the nucleotide sequences of genes. With the complete sequencing of the genomes of humans and other species, it will be possible to readily identify most if not all of the protein kinases.

From knowledge of the primary structure of a protein, it is feasible to produce nucleotide or antibody probes that are specific for that protein. Antibodies can be generated against the full length-expressed protein or portions. An effective strategy is to identify a region of about 10 to 20 amino acids that are extremely well conserved in that protein in diverse species, but which does not appear in other proteins. Antibodies generated, for example in a rabbit or mouse, against a synthetic peptide based on this amino acid sequence will cross-react with the full-length protein that contains this sequence, with little or no cross-relativity with other proteins. With the knowledge of the primary structures of all the protein kinases and other proteins encoded by mammalian genomes, specific antigen peptides can be designed to elicit the production of antibodies against all of the protein kinases.

Most protein kinases appear to be activated as a consequence of their own phosphorylation by upstream kinases or by self-phosphorylation (termed autophosphorylation). Phosphorylation cannot be monitored by nucleic acid-based approaches. However, phosphorylation of a protein can produce marked changes in its mobility on electrophoresis gels which are designed to act as a molecular sieve. Of such techniques, sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE) has become the standard method for separation of proteins on the basis of their size for analytical and preparative purposes. This technique relies of the sieving effect of the gel when proteins coated with negatively-charged detergent (eg. SDS) are drawn through the gel in an electric field. Smaller sized proteins are able to migrate through the gel faster than larger sized proteins. Proteins that differ by as little as a few hundred Daltons can be resolved by this method. Protein staining methods permit the visualization of discreet proteins in the gel as individual bands in a bar code like pattern. When these proteins are transferred from the gel onto a nitrocellulose membrane, the locations of specific proteins can be identified with antibodies in an immunoblotting procedure referred to as Western blotting (see: Towbin; U.S. Patent No. 4,452,901).

Most proteomic analytical methods are based on two dimensional (2D) gel electrophoresis by the standard method of Dr. Patrick O'Farrell described nearly 20 years ago. The 2D gel technique initially involves the separation of proteins in a first dimension based on their intrinsic charge in a pH gradient within a isoelectric focusing gel (typically a tube gel). Proteins migrate through the isoelectric focusing gel in the presence of an electric field until they encounter a pH at which the protein no longer possesses an electric charge. This pH is known as

the isoelectric point of a protein, and it is a distinguishing characteristic. Following electrophoresis in the first dimension, the isoelectric focusing gel is applied length-wise to the top of a molecular sieve gel such as a SDS-PAGE gel, and electrophoresis is continued into the second dimension. When the 2D gel is stained with sensitive-dyes (eg. based on silver reagent), the various proteins inside a cell can be visualized as resolved spots. The greater amount of a given protein within a cell sample, the larger and darker its specific spot appears. Several thousand proteins can be distinguished from one and another by this technique. If the protein samples have been obtained from cells that have been incubated with radioactive ^{32}P-phosphate, then the 2D gel can be exposed to x-ray film, and the phosphoproteins can be specifically detected. The more that a protein is phosphorylated or prevalent, the larger and more intense the spot on the x-ray film. The silver-staining of a 2D gel can be used to track the expression of proteins and their covalent modification by phosphorylation.

Public databases have been created that allow the identification of over a thousand different proteins on 2D gel proteomic maps. However, the positions of scarcely more than a dozen protein kinases are available. This reflects the fact that protein kinases are present at very minute levels in cells, and are often undetectable by even such sensitive protein dyes as silver-stain. Typically, transduction proteins are expressed at a hundred- to a thousand-fold lower levels than structural proteins and metabolic pathway enzymes. Therefore, it has often been necessary to incorporate selective enrichment techniques such as antibody-based purification as a preliminary step prior to 2D gel electrophoresis.

Proteomic analysis of kinases has evolved to date in much the same fashion as techniques for resolution of other proteins. One dimensional immunoblot techniques have been employed in which proteins from a smooth muscle homogenate are separated on an SDS-PAGE gel and then subjected to Western blotting followed by detection using a single polyclonal or monoclonal (MAB) anti-kinase antibody per blot (see: H. Togashi *et al.* (1997) "Quantitative Immunoblot Analysis of PKC Isoforms Expressed in Airway Smooth Muscle" Am. J. Physiol. 272 (Lung Cell. Mol. Physiol. 16): L603-L607). Conventional wisdom is that simultaneous use of multiple antibodies on a single immunoblot necessitates the use of MAB's (see: Coates, S.R. *et al.*; EP 025384 published January 27, 1988). Furthermore, 2D gel electrophoresis is thought to be the preferable technique for resolving complex protein mixtures. For example, Sanchez, J.C. *et al.* report the use of a mixture of nine MAB's for detection of different proteins indicative

of oncogene expression (including the kinase MEK-1)on a single immunoblot produced from 2D electrophoresis ("Simultaneous Analysis of Cyclin and Oncogene Expression Using Multiple Monoclonal Antibody Immunoblots" (1997) Electrophoresis 18:638-641).

While it is possible to visualize some protein kinases on 2D gels by immunoblotting techniques, we have determined that in most cases, a maximum of only four or five protein kinases can be detected at a time by Western blotting of 2D gels with mixtures of protein kinase-specific antibodies. Furthermore, we have found that recovery of most protein kinases from a first dimension pH gradient gel, is less than 10 %. That means that 90 % or more of the protein kinases do not enter the second dimension gel and are therefore unresolved.

Summary of Invention

This invention provides a method for detection of multiple kinases or multiple kinase substrates, whereby the presence and phosphorylation state of a large number kinases and/or kinase substrate proteins may be tracked in a single sample. This method comprises:

(a) obtaining a sample to be tested for kinase or kinase substrate content;

(b) optionally performing one or more of:

(i) addition of at least one protein phosphatase to dephosphorylate proteins in the sample;

(ii) inactivating protein phosphatase in the sample;

(iii) addition of at least one kinase and ATP to the sample; and

(iv) inactivating protein kinase in the sample.

(c) performing SDS-PAGE on the sample to produce a pattern of separated kinase or kinase substrate moieties from the sample;

(d) transferring the pattern to a membrane;

(e) treating the membrane with a panel of anti-kinase or anti-kinase substrate antibodies; and

(f) detecting the presence of antibodies from the panel bound to kinases or kinase substrates on the membrane.

When the method of this invention is employed to detect kinases in a sample either to elucidate the kinase profile of a tissue or cell type or to identify novel kinases, it is preferable that the panel of anti-kinase antibodies comprise polyclonal antibodies rather than MAB's. This departure from conventional wisdom increases the likelihood that new kinase proteins will be detected in the sample since polyclonal anti-kinase antibodies generally exhibit greater cross-reactivity to kinases as compared to anti-kinase MAB's. Despite the use of polyclonal antibodies, the panel may comprise from 2 to about 100 antibodies.

While (c) and (d) above in combination, is similar to standard Western blotting procedure, it is preferable that the electrophoresis gel be constructed to increase the likelihood that proteins will exhibit "band shift" between phosphorylated and dephosphorylated states. Typically, a protein will display reduced migration during SDS-PAGE when the protein is in a phosphorylated state. The reduced mobility may be as much as 1-5 kDa and this separation is enhanced by using a gel with a higher than normal acrylamide content and a lower than normal bisacrylamide content. While content of these gel components is normally adjusted to suite electrophoretic conditions and the average size of proteins to be separated, a 12.5% acrylamide/0.4% bisacrylamide gel is often suitable to achieve separation of phosphorylated and dephosphorylated kinases.

This invention also provides methods and apparatus whereby one or more values representing one or more kinases or kinase substrates are processed to identify correlations between individual kinases, kinase substrates and cell types or states. The method of this invention for detecting kinases or kinase substrates may be used to produce a series of such

values collectively referred to herein as a "profile". These profiles may be determined by correlation of specified parameters including presence or absence of a protein or substrate, density of spots or bands detected (for example, by densitromeric measurement), antibody specificity, state of phosphorylation, cell type and disease state. For example, densitromeric readings may be conveniently taken and digitized by available equipment. Values pertaining to various parameters as described above may be manipulated mathematically and correlated using appropriate computer equipment. The method of this invention provides an output value or values indicative of the relationship between selected protein kinases or kinase substrates. Such output is useful for identification of cell types or states (e.g. in diagnostic applications), for monitoring of treatment of a patient, for drug discovery and for assessing putative drug activity.

Apparatus of this invention includes machine readable memory means in which values as determined by this invention are stored. Such apparatus includes a computer programmed to correlate and/or store values determined by this invention. Such apparatus may include means for measuring and/or digitizing gels produced according to this invention.

Brief Description of the Drawings

Figure 1, shows gels presenting comparison of the multi-kinase immunoblotting patterns of different rat tissues. Electrophoresis of detergent solubilized lysates prepared from rat brain (A), heart (B) and skeletal (C) muscle was performed, and the positions of various protein kinases was visualized by ECL detection. Protein kinases of smaller size migrated correspondingly closer to the bottom of the SDS-PAGE gel. Each of the 18 strips derived from each SDS-PAGE gel were probed with different panels of protein kinase antibodies.

Figure 2, shows a gel demonstrating effects of anti-IgM treatment for 5 min on protein kinases in the human Ramos B cell line. Electrophoresis of detergent solubilized lysates prepared from Ramos cells untreated (-) or exposed (+) to anti-IgM polyclonal antibody for 5 min was performed in alternating lanes, and the positions of various protein kinases was visualized by ECL detection. Each of the 14 paired strips derived from two SDS-PAGE gels were probed with different panels of protein kinase antibodies.

Figure 3A. Fig. 3A are gels showing differential effects of kinase inhibitors on band shifting of selected protein kinases. Electrophoresis of detergent solubilized lysates prepared from human ovarian surface epithelial cells were untreated (Lane 1) or exposed to 20 ng/ml of human hepatocyte growth factor (HGF) in the absence (Lane 2) or presence of PD98059 (Lane 3), SB203580 (Lane 4), LY294002 (Lane 5) or rapamycin (Lane 6) was performed. The effects of these treatments on the positions of Erk1 and Erk2 (Panel A), p38 Hog MAP kinase (Panel B), PKB1 (Panel C), PKB2 (Panel D) and S6 kinase (Panel E) as visualized by ECL detection are shown. The phosphorylated and band shifted forms of these kinases are denoted with a "p" before their name. The partial structures of the protein kinases pathways in which these enzymes operate and the known sites of action of these drugs are shown in Fig. 3B.

Figure 4, are gels showing detection of known kinases and putative kinases in normal and tumor breast tumour biopsy samples of four human patients. Detergent solubilized lysates prepared from tumour (T) and adjacent control (C) breast tissue were subjected to multi-kinase profiling. In the left (A) panels, the increased levels of p38 MAP kinase, protein kinase B-α (PKBα), casein kinase 2 (CK2), protein kinase G (PKG) and cyclin-dependent kinase 8 (Cdk8) in the tumour samples is evident. Five of 12 proteins that were demonstrated to be elevated in tumours and not yet known for their identity are shown on the right (B) in Figure 4.

Figure 5 is a Western Blot showing separation of Erk1, Erk2 and protein kinase C-β by 2D gel electrophoresis. Detergent solubilized rat brain extract (1 mg protein) was subjected to isoelectric focusing and SDS-PAGE. In the left most lane, 200 μg of the brain extract was directly applied to the same SDS-PAGE gel. Following 2D gel electrophoresis, the proteins were transferred to a nitrocellulose membrane, which was probed with antibodies for Erk1, Erk2 and PKC-β.

Figure 6 is a chart showing examples of protein kinase pathways involved in nitrogenic and stress signalling.

Detailed Description

In this specification, the term kinase refers to those members of the class of enzymes that catalyze a chemical reaction in which a phosphate group is transferred from adenosine triphosphate (ATP) to a recipient protein. In the content of this specification, such a recipient protein is termed a "kinase substrate". This chemical reaction is called protein phosphorylation and is a reversible process with dephosphorylation being catalyzed by enzymes known as protein phosphatases. Kinases may be found in all organisms. In this specification, testing for kinase or kinase substrate content means determining the presence of at least one kinase or kinase substrate in a sample. Preferably the phosphorylation state of the kinase or kinase substrate will also be determined. In this specification, a kinase or kinase substrate moiety is a protein having the characteristics of a kinase or kinase substrate which occupies a single position after electrophoresis in a SDS-PAGE gel.

The following is a description of representive examples of different protein kinase pathways and their ability to cross-talk into different networks. This invention provides means to elucidate the kineomes and the interpretation of the various kinases that exist in a cell and their interaction within a kineome.

MAP kinase-dependent pathways

Mitogen-activated protein (MAP) kinase-dependent pathways have become a paradigm for how protein kinase pathways are constructed and operate. MAP kinases are a family of ubiquitously distributed, proline-directed protein-serine/threonine kinases involved in various signalling pathways that mediate cell growth, differentiation, transformation, cellular stress responses and apoptosis [1-7] (Fig. 6). The best characterized of the MAP kinases are 44-kDa Erk1 and 42-kDa Erk2. These isoforms are activated by more than 50 different extracellular proliferative stimuli that act through growth factor receptor-tyrosine kinases, hematopoietic receptors and seven transmembrane domain/trimeric G protein-coupled receptors [1-10]. They can phosphorylate various proteins including myelin basic protein (MBP), Rsk (ribosomal S6 protein kinase) and the transcription factor Elk1. For the maximum activation of Erk1 and Erk2, double phosphorylation within a Thr-Glu-Tyr motif located just before the conserved kinase subdomain VIII "APE" region by dual specificity kinases known as Mek1 and Mek2 is required. The proto-oncogene-encoded G protein Ras mediates Erk1 and Erk2 activation. The adapter protein Grb2, when bound to mitogen receptors, permits translocation of the Ras guanine-nucleotide exchange protein SOS to the plasma membrane where it can activate Ras by promoting its binding to GTP. Ras recruits the protein-serine/threonine kinase Raf1 to the plasma membrane, where it is activated via phosphorylation by other protein kinases including protein kinase C (PKC) [11-13] and Src family tyrosine kinases [11]. Raf1 directly phosphorylates and activates Mek1. Other protein kinases, such as Mek kinases (Mekk1, 2, 3 and 4) [14,15], Mos [16,17], and Cot (Tpl2) [18-21], have also been shown to phosphorylate and activate Mek1 *in vitro*, but apart from Mekk3, these Mek kinases probably target other Mek isoforms under physiological circumstances. Many other MAP kinase-like enzymes have been detected in mammals, including p38 Hog (α,_β,_γ and_δ) [22], Hera kinase [23], Erk3 [24], Erk4, Erk5 (alias Bmk) [25], and Jnk (alias SAPK-_α, β and γ) [26,27]. Most of these other MAP kinases are primarily implicated in stress signalling.

Jnk is activated in response to environmental stresses such as heat shock [44], hyperosmotic conditions [28,29], UV radiation [29,30], protein synthesis inhibitors such as anisomycin [31] and cycloheximide [27] as well as proinflammatory cytokines like TNF-α [29,30]. c-Jun is the important physiological target of this kinase [32-34]. Jnk are activated via phosphorylation of Thr and Tyr residues within a TPY motif located before their kinase subdomain VIII region [32]. This is catalyzed by a Mek-like kinase called Sek1 (Mkk4) [31,35]. Sek1 is activated in response to many but not all of the same stimuli that activate SAP kinases [31]. Mkk7 has also been shown to activate Jnk [36-39]. Sek1 is stimulated following phosphorylation by the 78-kDa form of Mekk1[14,40,41], which is generated by caspase 3 cleavage of a larger 196-kDa form of this kinase. Mekk1 phosphorylation of Sek1 occurs on Ser-220 and Thr-224 just upstream of the kinase subdomain VIII region [41]. Mekk1 itself is activated by phosphorylation of Thr-560 and Thr-572 located prior to subdomain VIII, but this is indirectly mediated through p21-activated kinases (Pak) and PKC [42]. Three related kinases of the Pak family have been cloned and shown to be activated by the Rho family G proteins Rac1 and Cdc42 [43,44].

These G proteins have been implicated in the membrane ruffling and actin cytoskeletal changes that are characteristic of transformed cells.

The p38 Hog MAP kinases are tyrosine phosphorylated and activated in response to a wide range of stress stimuli including hyperosmolar medium [22], Taxol [22], heat shock [45], arsenite [45], lipolysaccharide [46], interleukin 1 [47,48] and TNF-α [47,48], but not in response to phorbol ester. Four distinct p38 Hog isoforms (α, β2, γ (alias Sapk3 or Erk6) and δ (alias Sapk4)) have been characterized in mammalian cells and they exhibit very similar properties [49,50]. They are all capable of phosphorylating MBP, MAPKAPK2, MAPKAPK3, Mnk1, ATF-2, Elk-1 and Sap-1, although there are some differences in substrate preferences. They induce the phosphorylation of the 27-kDa heat shock protein via the intermediary kinase MAPKAPK2 [51-53]. The mammalian p38 Hog isoforms differ in their tissue distribution, with α and β2 broadly expressed, γ found primarily in muscle and brain, and δ expressed predominantly in salivary gland, pituitary gland, adrenal gland and placenta [54]. The p38 Hog isoforms are sensitive to inhibition by pyridinyl imidazole compounds like SB203580. The p38 Hog MAP kinase has a similar dual-phosphorylation site Thr-Gly-Tyr (TGY) motif N-terminal to catalytic subdomain VIII as found in other MAP kinases. Mkk3 and Mkk6 have both been shown to phosphorylate these sites to activate p38 Hog isoforms [35]. Mkk3 is stimulated in COS cells in response to UV radiation, osmotic stress, TNF-α and interleukin-1, but not by EGF or phorbol myristate actetate (PMA) [35]. Like other Mek isoforms, Mkk3 and Mkk6 feature two phosphorylation sites (Ser-187 and Thr-191 in Mkk3) that are necessary for activation. One of the kinases implicated in the phosphorylation and activation of Mkk3 is Tak1 [55].

The intervening steps that transduce signals from cell surface receptors for pro-inflammatory agents and other stress stimuli into Mekk and Mek isoforms are obscure. Intermediary protein-serine/threonine kinases such as Lok, Mst1, Mst2, Nik, Sok, Khs, Gck, Hpk, Mlk3 and Zpk are among a plethora of possible candidates, but the precise positioning of these kinases in the signalling pathways remains ambiguous [56] (Fig. 6). It is possible that some of these kinases directly regulate other MAP kinase homologues such as Erk3 and Erk5, for which much less is known.

PI 3-kinase

The phosphatidylinositol 3-kinase (PI 3-K), protein kinase B (PKB) and p70 S6 kinase ($p70^{S6K}$) signaling pathway is implicated in multiple cellular functions including growth factor signal transduction.

PI 3-K was first identified as a heterodimer of 110 kDa and 85 kDa proteins [57]. p110 is a catalytic subunit which contains a C-terminal catalytic domain, an adjacent lipid kinase unique domain, a Ras binding domain and an N-terminal region which binds to the p85 adapter protein. The p85 adapter protein contains two SH2 domains and an N-terminal SH3 domain. The function of p110 is to catalyze the transfer of the γ-trisphosphate of the ATP to the D3 position of phosphoinositides (PtdIns) [58]. In addition to this lipid kinase activity, the p110 subunit also contains a C-terminal region which is related to subdomain VI and VII of protein kinase. This may be the structural basis for the protein-serine kinase activity of p110 towards the p85 adapter protein [59].

Since the identification of the p110/p85 heterodimer, a large collection of p110 and p85 homologues have been identified [60,61]. Mammalian p110 homologues can be divided into 4 groups based on their structural and functional characteristics. These are: 1) the classic PI 3-K group including p110α, β and γ; 2) Vps34 [62,63]; 3) PI 4-K [64,65]; and 4) the mammalian target of rapamycin (mTOR) subfamily including mTOR [66-68], DNA-dependent protein kinase (DNA-PK) [69] and ataxia-telangiectasia (ATM) [70].

A major upstream signaling pathway which leads to the activation of PI 3-K is growth factor receptor initiated signaling which is exemplified in the insulin signal transduction [71]. In this case, PI 3-K is translocated to membrane through interaction between p85 subunit and tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1). In addition, PI 3-K may function as a downstream effector of Ras [72]. Furthermore, PI 3-K may also be activated by heterotrimeric G protein-mediated signaling, and by protein tyrosine kinases such as Src and focal adhesion kinase [73-75].

One of the approaches to investigate the downstream signaling events of PI 3-K has

been to identify cellular components which interact specifically with D3 phosphoinositides. PtdIns 3,4,5-P_3 binds specifically to the SH2 domain of the Src and p85 adapter subunit of PI 3-K [76]. In addition, there is evidence of the activation of PKC isomers by PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3 [77,78]. However, the functional relevance of the interaction between phosphoinositides and signaling proteins is most evident in the case of the activation of protein kinase B (PKB) following specific interaction between the pleckstrin homology (PH) domain of PKB and PtdIns 4,5-P_2 and PtdIns 3,4,5-P_3 [79].

Protein kinase B

PKB is named for its sequence homology with protein kinase C and protein kinase A (also known as Rac-PK (for related to the A and C kinase) and Akt). To date, four members of the PKB family have been identified including PKB-α, β1, β2 and γ. All 4 PKB share a similar primary structure constituting of two functional domains, an N-terminal PH domain and a C-terminal catalytic domain.

PKB is activated *in vivo* by a variety of growth stimuli including platelet-derived growth factor (PDGF), insulin, epidermal growth factor (EGF) and nerve growth factor (NGF), and this appears to be mediated by PI 3-K [80,81]. The mechanism of PI 3-K-mediated activation of PKB involves a PH domain-mediated lipid binding and specific phosphorylation [58,82]. The PH domain of PKB has been shown to bind D3 phosphoinositides with particularly high affinity to PtdIns 3,4-P_2 [83,84]. Full activation of PKB requires phosphorylation of Ser-473 and Thr-308 residues within the catalytic domain of PKB [85]. PtdIns 3,4,5-P_3-dependent protein kinase-1 (PDK1) phosphorylates the Thr-308 of PKB, resulting in an increase in kinase activity of over 30-fold [86]. The activity of PDK1 is also potentiated *in vitro* by low micromolar concentrations of the PtdIns 3,4-P_2 and PtdIns-3,4,5-P_3 under the conditions where a direct activation of PKB by the phosphoinositides is not observed. These findings indicate an indirect effect of phosphoinositides on PKB with PDK1 as an intermediate.

PKB can also be activated in a PI 3-K independent manner. In COS-7 cells, PKB was activated by heat and osmotic shock through a PI 3-K independent pathway [87]. UV, heat and osmotic shock are known to cause strong activation of p38 HOG1. It has been suggested that p38 HOG1 functions as an alternative upstream kinase mediating stress-induced activation of PKB. This is further supported by the observation that Ser-473 of PKB can be phosphorylated *in vitro* by MAPKAPK-2, a direct target of p38 HOG1 [85].

The only known *in vivo* substrate for PKB is glycogen synthase kinase-3 (GSK3). Upon insulin stimulation, PKB phosphorylates GSK-3 [88]. This phosphorylation led to the inactivation of GSK3, which may be responsible for some of the insulin-induced metabolic events [89]. There is also evidence supporting the possibility that p70 S6 kinase ($p70^{S6K}$) is a downstream target of the PI 3-K/PKB pathway, although it is unlikely that $p70^{S6K}$ is a direct target of PKB [90].

S6 kinase

$p70^{S6K}$ was first discovered for its role in the phosphorylation of S6 protein of the 40S ribosomal subunit upon growth factor stimulation [91]. It was found later to be activated by almost all the known mitogenic stimuli including growth factor, cytokines, phorbol esters and oncogene products [92]. In addition, $p70^{S6K}$ can also be activated by inhibitors of protein translation including cycloheximide and anisomycin [93].

Four isoforms of $p70^{S6K}$ have been identified to date [94,95]. These are the cytoplasmic p70 and nuclear p85 isoforms, and two cytoplasmic p54 isoforms. The primary structure of $p70^{S6K}$ can be divided into 4 functional domains including an acidic N-terminal sequence, a catalytic domain, a basic C-terminal auto-inhibitory domain and a linker sequence that bridges the catalytic domain and the C-terminal sequence. These are designated as domains I, II, IV and III, respectively [96]. $p70^{S6K}$ is phosphorylated at basal level in quiescent cells [92]. Following mitogenic stimulation, it is further phosphorylated at multiple sites [96]. To date, at least 10 inducible phosphorylation sites have been identified. The molecular mechanism for phosphorylation-induced activation of $p70^{S6K}$ and the upstream kinases responsible for the phosphorylation of individual sites are not understood.

The current understanding of $p70^{S6K}$ has to a major extent benefited from the discovery of an immune suppressive drug, rapamycin. Rapamycin is known for its effect in blocking cell cycle progression, and for its inhibitory effect on the activation of $p70^{S6K}$ induced by virtually all the known activators of the kinase [97-99]. In addition, rapamycin is highly specific. It does not inhibit the activity of Rsk1, a kinase which is closely related to $p70^{S6K}$ [97]. The fact that rapamycin causes rapid inhibition of $p70^{S6K}$ activation induced by a variety of stimuli indicates that the effect of rapamycin is exerted at a target proximal to $p70^{S6K}$ [100]. This target was first identified in yeast as target of rapamycin (TOR) and later on in mammals as mTOR (also known as FRAP and RAFT) [101,102]. It is now known that rapamycin binds to a cytoplasmic protein called FKBP12 and the complex then binds to mTOR, thereby exerting the inhibitory effect on $p70^{S6K}$ [103,104]. mTOR is a member of PI 3-K family [101]. Brown *et al.* (1995) [100] reported an *in vitro* autophosphorylation of mTOR, and this intrinsic protein kinase activity was sensitive to rapamycin and was required for the mTOR-mediated regulation of $p70^{S6K}$. mTOR has not been shown to directly phosphorylate $p70^{S6K}$, therefore, it is unlikely to be an immediate upstream kinase directly involved in the phosphorylation of $p70^{S6K}$. A recent model proposed by Pullen and Thomas predicates that mTOR is involved in the opening of $p70^{S6K}$ from its closed conformation, thereby allowing subsequent phosphorylation of some of the critical residues, and these phosphorylation events may be mediated by yet unidentified upstream kinases [96]. In addition to its role in the activation of $p70^{S6K}$, mTOR has also been shown to mediate insulin-induced phosphorylation of (eIF)-4E-binding protein (4E-BP, also known as PHAS-1), a repressor of eukaryotic translational initiation [105].

PI 3-K and PKB are upstream signaling components in mitogen-induced activation of $p70^{S6K}$, since wortmannin treatment inhibits insulin- or PDGF-induced activation of PI 3-K as well as $p70^{S6K}$ [106,107]. In addition, the expression of constitutively active PI 3-K activates $p70^{S6K}$ [108]. Furthermore, the expression of Gag-PKB fusion construct which is homologous to the *v-akt* oncogene activates $p70^{S6K}$ [80]. These observations indicate a mitogenic pathway involving PI 3-K, PKB and $p70^{S6K}$. However, PKB does not directly phosphorylate $p70^{S6K}$, and PKB-mediated activation of $p70^{S6K}$ is sensitive to rapamycin. Therefore, PKB is either upstream of mTOR or shares a common downstream target with mTOR.

In addition, $p70^{S6K}$ may also be regulated by upstream signaling pathways involving protein kinase C (PKC) [109]. This is based on the observation that phorbol ester caused the activation of $p70^{S6K}$. This phorbol ester-induced activation of $p70^{S6K}$ is sensitive to inhibition by rapamycin. Phorbol ester-induced activation of $p70^{S6K}$ is less sensitive to wortmannin than to rapamycin. In addition, phorbol ester activated PKB very weakly if at all [110]. These observations indicate that PKC may function upstream of mTOR and in parallel with the PI 3-K/PKB pathways. In addition, Cdc42 and Rac1 are also implicated in the activation of $p70^{S6K}$ [111].

The involvement of $p70^{S6K}$ in cell cycle regulation was first indicated by the observation that mitogen-induced activation of $p70^{S6K}$ and progression through the G1 phase of the cell cycle were inhibited by rapamycin treatment and by micro-injection of anti-$p70^{S6K}$ neutralizing antibody [99,101]. In addition, $p70^{S6K}$ also plays an important role in regulating the translation of a class of mRNA which contain an oligopyrimidine tract at its transcriptional start (5'TOP)[112]. The 5'TOP group of mRNA transcripts encode all the known ribosomal proteins and some of the elongation factors.

Samples used to be tested for kinase or kinase substrate content may be any cell or tissue homogenate, extract or other such sample which has been processed to purify or partially purify kinases or kinase substrates in the sample. This invention is particularly suitable for testing patient biopsy samples. Such samples may be manipulated to increase prevalence of desired cell types in the sample. The sample will be typically prepared for electrophoresis using standard techniques, employing appropriate buffers which may contain various inhibitors or enzymes. For example, protease inhibitors may be present to reduce protein degradation on the sample. Where the sample is to be tested for kinase substrate content, it may be desirable to add one or more protein phosphatases to dephosphorylate substrates which may already exist in a phosphorylated state in the sample. The protein phosphatase will then be inactivated with an appropriate phosphatase inhibitor (eg. β-glycerophosphate, sodium fluoride or sodium orthovanadate) and a selected protein kinase or mixture of protein kinases is then added to the sample to phosphorylate those substrates present which are specific to kinase added to the sample. Alternatively, endogenous kinases in the sample may be relied upon to phosphorylate dephosphorylated substrates in the sample.

SDS-PAGE employed in this invention is gel electrophoresis performed in a single dimension, typically using a slab shaped gel or a series of tube gels. The gel may be constructed and used employing standard methods, electrophoresis buffers and electrophoresis equipment. The gel may comprise a stacking gel and a separation gel. Commercial kits and equipment are available for performing SDS-PAGE. Preferable contents of the separation gel range from 10% to 15% (acrylamide) and 0.2 to 2% (bisacrylamide). For separation of kinases, an electric current will typically be applied to the gel until protsins with a molecular mass of less than about 25-27 kDa are eluted from the bottom of the gel as protein kinases do not have a molecular mass less than the latter amount. Once electrophoresis is complete resulting in a pattern of separated protein moieties in the gel, the pattern is transferred to any membrane (eg. nitrocellulose, PVDF, nylon, etc.) that is suitable for use in the Western Blotting technique. Transfer is typically done by standard electro-transfer techniques. One the pattern is transferred to the membrane, the membrane may be cut into strips each of which will typically contain a pattern separated from a single sample (eg. a test sample or a control sample).

Once the electrophoresis and Western blotting aspects of this method are complete, the resulting membrane or membrane strips are probed with a panel of different antibodies that react with distinct categories, subsets, isoforms, etc. of protein kinases or kinase substrates. The panel may be applied in one step as a mixture of antibodies or, the antibodies may be applied sequentially to the membrane. Binding of such antibodies to moieties present on the membrane is then detected using any suitable immunoassay procedure (eg. see: Stites and Terr (eds) "Basic and Clinical Immunology", (7 ed) 1991). A particularly suitable procedure is to treat the antibodies in the panel as primary antibodies in a "sandwich" type assay. Unbound primary antibodies are washed away or otherwise removed. The membrane is then treated with secondary antibodies which are reactive with the primary antibodies. The secondary antibody may be bound to a detectable label or fused with an enzyme. Secondary antibody bound to primary antibody is detected by observing the label or the activity of the fused enzyme. Suitable labels and enzymes are known in the art and include magnetic or coloured beads, fluorescent dyes, radiolabels. horseradish peroxidase, alkaline phosphatase, etc. The enzyme linked sandwich type assay (ELISA) is a particularly suitable methodology for use in this invention.

Antibodies for use in the method described herein may be obtained commercially or prepared using standard techniques. A variety of anti-kinase and anti-kinase substrate polyclonal antibodies are commercially available from various sources, including the following:

Biomol Research Laboratories, Inc. (Plymouth Meeting, Pennsylvania)
Biosource International, Inc. (Camarillo, California)
Promega Corporation (Madison, Wisconsin)
Santa Cruz Biotechnology (Santa Cruz, California)
Sigma (Saint Louis, Missouri)
StressGen Biotechnologies Corp. (Victoria, British Columbia)
Transduction Laboratories (Lexington, Kentucky)
Upstate Biotechnology Inc. (Lake Placid, New York)
Zymed Laboratories Inc. (South San Francisco, California)

Antibodies to new kinases may be prepared as described below. Typically, new kinases are partially purified by techniques such as column chromatography and SDS-PAGE. Microsequencing of partially purified kinases permits comparison to known kinases and possible development of immunological techniques for recovery of more of the new kinase by making use of cross reactivity with known antibodies. Antibodies can be raised against protein kinases or substrates in various host animals, including but not limited to cattle, horses, rabbits, goats, sheep and mice. Polyclonal antibodies can be obtained from immunized animals and tested for specificity using standard techniques. Alternatively, monoclonal antibodies may be prepared using any technique that provides for production of antibody molecules by continuous cell lines in culture, including the hybridoma technique of Kohler and Millstein, the human B-cell hybridoma technique, and the EBV-hybridomain technique. Alternatively, techniques for the production of single chain antibodies and antibody fragments that contain specific binding sites for a protein kinase or substrate may be generated by known techniques and employed in this invention. Such fragments include $F(ab')_2$ fragments that may be generated by digestion of an intact antibody molecule and Fab fragments that may be generated by severing dissulfide bridges in $F(ab')_2$ fragments or through the use of Fag expression libraries.

Preferably, none of the antibodies in a given mixture to be used as a panel in this invention will cross-react with proteins that overlap in size. This may compromise interpretation of the experimental findings. Each antibody panel mixture should be blended to avoid such overlaps. Furthermore, every mixture should be adjusted for the concentration of each antibody so that there is optimal detection of the individual target kinases in diverse cell and tissue samples.

Following incubation of the strips with different mixtures of primary antibodies, the strips are incubated with a secondary antibody (eg. a goat antibody that recognize rabbit antibody) that reacts with the primary antibody. The secondary antibody is fused with an enzyme (eg. alkaline phosphatase or horse radish peroxidase) to facilitate detection of the positions of the primary antibody, to which it binds by producing a light emission in an enzymatic reaction. The separate strips may be reassembled to appear in the order of the original membrane. The reassembled membrane may be subjected to enhanced chemiluminescence (ECL) and exposure to x-ray film or detected by a phosphoimager

(eg. Fluor-S Max Multi-imager from Bio-Rad Laboratories). In this indirect manner, the original positions of resolved protein kinases can be visualized as dark bands on a transparent background. The intensity of the bands can be quantitated by densitiometric analysis. In many cases, quantitation of the amounts of a given protein kinases in the upper, phosphorylated form and the lower dephosphorylated form can provide an accurate measurement of how much of the kinase is in the inactive and active states.

The method described herein offers advantages over standard 2D gel proteomic methods. This technique can be applied to any cell or tissue sample. No prelabelling with radioisotopes is necessary, because kinase detection is based on immunoreactivity. The technology could be adapted for wide scale diagnostic applications because the patterns of protein kinase expression are stable for periods of up to six hours before an organ is subjected to fractionation and freezing, providing the organ is stored during this time over ice. This procedure can be carried out within two days from start to finish. By contrast, the 2D gel electrophoresis approach is extremely laborious, much more difficult to render and takes at least twice the time. This method provides the ability to compare multiple samples side by side. Whereas two or more samples can be analyzed on the same 1D gel, a 2D gel can only be used for a single sample. It is more difficult to compare two different samples by the 2D gel route, because of potential variations in the setting up, running and analysis of separate 2D gels.

One of the reasons why 2D gel electrophoresis has become the industry standard for proteomic analysis is the remarkable resolving power of the method and potentially thousands of spots can be distinguished on a 2D gel. Most of these spots, however, are "fuzzy" in appearance and may be overlapping. The method described herein provides much tighter protein bands with a 2- to 4-fold better resolution in the SDS-PAGE size-separation dimension. With detection based on immunoreactivity, the background of metabolic enzymes and structural proteins is essentially eliminated. This background is problematic even for 2D gel maps of phosphoproteins, since a third of all the proteins inside of cells appear to be phosphorylatable.

In one exemplary embodiment, about 50 μg of a control cell extract from untreated or healthy cells is loaded on to a SDS-PAGE gel in odd numbered lanes. In adjacent, even

numbered lanes, equivalent amounts of experimental extracts are deposited. The latter samples are from cells that have been treated with a hormone or drug or that have been obtained from diseased tissue. The extracts may be prepared by homogenizing cells in buffer containing a detergent such as 0.5% Triton X-100™ and protein phosphatase inhibitors (to preserve the state of protein phosphorylation in the sample). The extracts are then subjected to ultracentrifugation to remove insoluble matter.

To optimize the detection of protein band shifts, the SDS-PAGE gel is precast with a higher than normal concentration of acrylamide and a lower than normal concentration of bisacrylamide. An electric current is applied to the slab gel until proteins with a molecular mass less than 27,000 Dalton are eluted from the bottom of the gel. The proteins remaining on the slab gel are then electro-transferred on to a nitrocellulose or PVDF membrane that traps the proteins. The membrane is cut into separate strips that each contain samples of the resolved proteins from both control and experimental cell extracts. Each strip is probed with a different mixture of primary antibodies (eg. from rabbit) that react with a distinct subset peptide or protein substrate by the protein kinase of interest. Each reaction is conducted in a separate tube or well of a microtitre plate.

One application of this invention is for the discovery of novel protein kinases. The following strategy should permit the rapid acquisition of protein kinase drug targets. The objective of this approach is to identify those protein kinases that demonstrate increased expression or phosphorylation in association with a disease state or in response to an extracellular signal such as mitogen, drug or stress factor. The approach is based on the following:

1. Antibodies developed for one protein kinase can cross-react with structurally related protein kinases.

2. A band shift of a cross-reactive protein on an immunoblot is due to phosphorylation, and increased phosphorylation is probably associated with activation of the kinase. Greater than 90% of the known protein kinases are phosphorylated in their active states. One of the exceptions is glycogen synthase kinase-3, which is inhibited when it is phosphorylated on

serine by protein kinase B. However, activation of glycogen synthase kinase-3 is still dependent on tyrosine phosphorylation of this kinase.

3. Proteins that cross-react with protein kinase antibodies and also bind to gamma-ATP-agarose beads have a very high probability of being protein kinases. This resin will capture many ATP binding proteins in addition to protein kinases but this procedure can purify kinase by up to 200-fold.

4. Proteins that autophosphorylate with [γ-^{32}P]ATP following immunoprecipitation with protein kinase antibody are likely to be protein kinases. Most antibodies are unsuitable for immunoprecipitation of proteins, and may require partial denaturation of the proteins. Denatured kinases would have little or nor autophosphorylating activity.

5. A combination of gamma-ATP-agarose, immunosorbent and fast protein liquid chromatophy column steps followed by SDS-PAGE permits rapid purification of an immunoreactive protein to allow for its identification by sequencing.

6. There is a likelihood that a protein kinase detected with antibodies is novel. Of the 2000 or so kinases expected to exist, only about a quarter have been fully sequenced. Nevertheless, partial cDNA sequences for most protein kinases are available in public and private EST cDNA sequence databases. Once a portion of the cDNA structure of the protein kinase gene is available, it is straightforward to obtain the complete nucleotide and amino acid structures of the gene and its protein with standard methodologies.

One of the beneficial outcomes of this invention is that unknown proteins which can cross-react with the kinase-specific antibodies are detected. Those unidentified proteins that change in their abundance or their phosphorylation state in response to a disease condition or treatment are worthy of closer analysis. If such proteins can be shown to bind to ATP-agarose or capable of autophosphorylation with radioactively labelled ATP, then there is a high probability that they are protein kinases. Moreover, it is possible to purify the protein so that it can be sequenced by the Edman degradation method or identified by mass spectroscopy of

trypsin digested fragments of the protein. Purification is best done using the antibody that was originally used to detect the putative kinase. If any part of the protein has been previously sequenced, it would be available in public or private protein sequence databases. A partial sequence in the human EST sequence database may be available. From this information, a full length cDNA sequence for the protein could be rapidly obtained using PCR-based techniques. This would be worthwhile if the cDNA sequence contained conserved kinase catalytic subdomain sequences. In this manner, novel protein kinases that display desirable characteristics (eg. increased expression in solid tumour relative to adjacent, normal tissue) can be detected and identified. If the inappropriate activity of such protein kinase is shown to contribute to the development of the disease, then they would be most valuable drug targets.

Initial detection of measurement of the activation of a protein kinase in the methods described herein is dependent on the detection of its band shift on SDS-PAGE gels. Phosphorylated forms often appear to be 0.5 to 2 kDa larger than their dephosphorylated counterparts. In a small number of cases, phosphorylation is indicative of inactivation of a kinase. A limited number of protein kinases do not exhibit a band shift change when they are activated. However, their *in vivo* substrates can display band shifts upon their phosphorylation. This can be exploited for the development of *in vitro* and *in vivo* substrate assays. Current approaches for high throughput screening of protein kinase inhibitors *in vitro* involve the use of radioactive [γ-^{32}]ATP and measurement of the incorporation of the radioactive phosphate into a peptide or protein substrate by protein kinase of interest. Each reaction is conducted in a separate tube or well of a microtitre plate generating high volumes of radioactive garbage.

There are many examples of proteins that are highly specific substrates of particular protein kinases; examples include glycogen phosphorylase for phosphorylase kinase, myosin light chain for myosin light chain kinase, eIF2α for PKR, MARCKS for protein kinase C, Erk1 and Erk2 for Mek1 and Mek2. Antibodies are commercially available for many of these substrates or may be produced as described above. Such antibodies may be used to probe for the phospho-states of the substrates, as revealed by their mobility on immunoblots of SDS-PAGE gels. These substrates would not have to be purified from crude cellular extracts for use in the protein kinase assays. However, since may of the substrates may already exist in

phosphorylated forms in cell extracts, it may be necessary to incubate the extracts with active preparations of protein phosphatases, which can be subsequently inactivated with phosphatase inhibitors prior to the kinase assays. With this method, a crude mixture of active protein kinases may be added to the phosphatase-treated cellular extract in a single tube, and the phosphorylation reaction can commence with the inclusion of non-radioactive ATP. Only catalytic amounts of protein kinases will be necessary, so any phosphorylated substrates that contaminate the preparation of protein kinases will be relatively minor compared to the amounts of the substrates in the phosphatase-treated cell extracts. Any kinases that contaminate the phosphatase-treated cell extracts would not be a concern, since they are actually desirable. It may be necessary to add a kinase preparation after phosphatase treatment of the substrate extracts, because many protein kinases are inhibited when they are dephosphorylated. After a short suitable incubation time, the reactions can be terminated by addition of SDS-PAGE sample buffer. Such substrate analysis can be performed as described above for protein kinases, except that panels of antibodies for the kinase substrates will be used in place of the kinase antibody panels. By this approach, the decreased mobility of the kinase substrates will be evident as band shift on the immunoblots in the absence of kinase inhibitors. The presence of specific protein kinase inhibitors would be revealed by the inhibition of the appearance of the upper bands.

In vitro substrate analysis according to the methods described herein would be ideal for the further characterization of compounds that have already been shown to display inhibitor activity toward a kinase and the selectivity of these compounds is in question. A distinct advantage of this method is that it would be easy to compare the findings with a substrate analysis *in vivo* assay performed using the same blends and concentrations of kinase substrate antibodies that work in the *in vitro* kinase assay. However, the analysis would be performed on extracts from cells that have been incubated with agonists that stimulate the kinases of interest. These cells would also be exposed to the compounds that exhibit inhibitory activity towards kinase *in vitro*. In this manner, the efficacy of these inhibitors could be evaluated in living cells.

The intensity of the signal that is generated for a protein kinase band may be readily quantified using known technologies. For example, quantification may be done by using a phosphorimager. This equipment can quantitate changes in band intensity in range of 1:100,000. Multiple exposures of X-ray films to ECL for detection of immunoreactive bands to compensate for any non-linearity of response of the film prior to quantitation by densitometric analysis could be performed as an alternative method.

Each immunoreactive band is assigned a set of parameters that includes its relative optical density, molecular weight and immunoreactivity. The relative optical density (R.O.D.) value of an immunoreactive protein band is based on the ratio of the intensity of that protein relative to the intensity of a protein kinase band that serves as an internal control. For example, the mitogen-activated protein (MAP) kinase Erk1 in 50 μg of rat brain cytosolic protein detected with Erk1-CT antibody could serve as such an internal control. Erk1 has been found to be one of the most uniformly expressed protein kinases in different rat tissues and diverse organisms. Alternative standards could be the zeta isoform of protein kinase C or the alpha isoform of p38 Hog MAP kinase. If a protein has the same intensity on a Western blot as Erk1 in rat brain, then it has an R.O.D. value of 100.

Another parameter is the molecular mass of an immunoreactive protein band, which is based on its migration on the SDS-PAGE gel relative to known molecular mass marker proteins such as phosphorylase, bovine serum albumin, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase and lysozyme.

A further paramet is the immunoreactivity of an protein band, which is somewhat selective, and particularly appropriate when the immuogen to which the antibody was originally developed is considered. For example, an antibody developed against the C-terminal 40 amino acids of the rat brain Erk1 isoform would be expected to immunoreact with the full-length 44 kDa form of Erk1 on Western blots of rat brain cytosol.

Determination of the structure of a protein kinase network is based on the following principles.

i. Modules of protein kinases have been highly conserved in the evolution of diverse eukaryotes. These modules mediate the transmission of information in signaling pathways.

ii. Protein kinases that operate within a common module would be typically coexpressed in cells found in different tissues and species.

iii. Protein kinase networks are formed from the interconnection of diverse protein kinase modules. This implies extensive cross-talk between signaling modules.

iv. An upward shift in a protein kinase band due to reduced mobility reflects its regulation, most likely marking its activation. If other protein kinase bands are similarly affected by the same set of cell stimuli, then they may operate within the same module.

v. The higher the correlation between two protein bands with respect to their coexpression and their band shifting in response to a wide diversity of stimuli, the closer that they operate within a common signaling module. Protein kinases that are not coexpressed and do not undergo coregulation, are not connected in the same pathway.

Determination of the architecture of a protein kinase network requires the examination of a wide range of different cell types and perturbations. Ideally, at least one hundred different experimental model systems will be analyzed. It is important that while extremely diverse model systems are explored, the methods and probes for the Western blotting analysis are uniformly consistent. By testing the regulation of the same group (e.g. 100) of different protein kinases in a several diverse model systems with a wide range of different perturbations, each immunoreactive band can be seperately compared with each of the other hundred or more immunoreactive bands for (1) the intensity of the signals and (2) whether the protein kinase are similarly altered in their phosphorylated states.

It is expected that a given protein kinase will be expressed to varying degrees in different cell types. The coexpression of two different protein kinases can be quantified by

comparison of the R.O.D. values dervied from the intensity of their immunoreactive signals. It will be useful to sum the intensities of the signals for the bands corresponding to the dephosphorylated and phosphorylated states of a given kinase for the coexpression parameter.

The coexpression quotient (C.E.Q.) of two protein kinases could be determined as follows. Upon examination of all of the model systems in which a particular protein kinase is investigated, the highest R.O.D. value is taken as a given value (e.g. 100), and all other R.O.D. values are expressed relative to this. Therefore, the highest level of expression would have a relative expression (R.E.) value of (in this example) 100 and the complete absence of the protein would have an R.E. value of 0. In each experimental model system, the difference in the R.E. values of the two proteins would be determined. These differences would be summed and then divided by the number (N) of model systems in which the two proteins were compared to derive the C.E.Q. value. The lower the C.E.Q. value, the higher the probability of a relationship between the two proteins.

By way of illustration, examples from different cell types are given to demonstrate the present invention. The following examples are not intended to be limiting of the invention.

Example 1

In this example, the kinase multi-blot analysis is used to probe for the presence of over 45 different protein kinases in soluble extracts prepared from the whole brain, heart and skeletal muscle of adult male Sprague-Dawley rats. The results demonstrate large differences in kinase expression patterns between these tissues.

Materials

Affinity-purified rabbit polyclonal or monoclonal antibodies and immunizing peptides used to raise these antibodies are listed in Table 1. These antibodies were either prepared or obtained commercially. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was obtained from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were obtained from Amersham Pharmacia Biotech, Inc. (Baie dUrfe, Quebec). Other reagents were obtained from Sigma-Aldrich (St. Louis, MI), unless otherwise stated.

Preparation of rat tissue extracts

Brains, hearts and hind leg tibial skeletal muscles from 50-day old male Sprague-Dawley rats were rapidly excised, after induction of anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg). The tissues were cut, rinsed with phosphate buffered saline at 4 °C, frozen in liquid nitrogen, and stored at -70 °C until use. The tissues were pulverized with 5 strokes of a liquid nitrogen-cooled hand French press and re-suspended in 10 volumes of ice-cold homogenization buffer containing: 20 mM MOPS, 15 mM EGTA, 2 mM Na_2EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin, and 1% Triton X-100. This was then sonicated with a Branson Probe Sonicator at 4°C with 3 x 30 s bursts. The homogenates were ultracentrifuged at 100,000 rpm (240,000 x g) for 15 min in a Beckman

TLA-100.2 ultracentrifuge at 4 °C. The supernatants were immediately frozen at -70 °C until subsequent analysis.

Table 1. Sources of antibodies.

	Kinase	Ab Name	Conc. (µg/ml)	Source & Catalog No. or immunizing peptide[1]
1.	Calmodulin-dependent kinase kinase	CaMKK-CT	0.2	StressGen KAP-CA001
2.	Calmodulin-dependent kinase 4	CaMPK4-NT	1.0	StressGen KAP-CA003
3.	Cyclin-dependent kinase 1 (cdc2)	Cdc2-CT	0.7	StressGen KAP-CC001
4.	Cyclin-dependent kinase 2	Cdk2	1.6	StressGen KAP-CC007E
5.	Cyclin-dependent kinase 5	Cdk5-CT	2.4	Upstate 06-258
6.	Cyclin-dependent kinase 6	Cdk6	0.8	StressGen KAP-CC008E
7.	Cyclin-dependent kinase -7	MO15-PCT	0.5	StressGen KAP-CC010E
8.	Cyclin-dependent kinase 8	Cdk8-NT	2.0	StressGen KAP-CC008E
9.	Casein kinase 1 α and ε	CK1 SG	1.0	StressGen KAP-ST103E
10.	Casein kinase 2 α	CK2α-III	1.2	StressGen KAP-ST010
11.	Cot (Tpl2)	Cot-PCT	0.5	EESEMLKRQRSLYIDGC[2]
12.	p43 and p45 MAP kinase homologues	Erk1-III	0.6	Upstate 06-183
13.	Erk1 and Erk2 MAP kinases	Erk1-CT	0.3	Upstate 06-182
14.	Erk5 MAP kinase (Bmk)	Erk5-PNT	4.0	SAEPPAREGRTRPHRC[2]
15.	Glycogen synthase kinase β	GSK3β-XI	2.0	StressGen KAP-ST002E
16.	Integrin linked kinase 1	ILK1 SG	1.0	StressGen KAP-ST203
17.	Kkialre Cdk-like kinase	Kkialre-CT	3.0	StressGen KAP-CC003
18.	Kinase-suppressor of Ras	Ksr1-CT	1.0	EKLPKLNRRLSHPGHFWKSC[2]
19.	MAP kinase-activated kinase 2	MAPKAPK2-PCT	1.0	StressGen KAP-MA015E
20.	MAP kinase kinase 1	Mek1-XI	2.0	Upstate 06-235
21.	MAP kinase kinase 3	Mkk3-CT	1.0	Upstate 06-615
22.	MAP kinase kinase 4	Mkk4-XI	1.6	Upstate 06-281
23.	MAP kinase kinase 5	Mek5-PNT	1.6	StressGen KAP-MA003
24.	MAP kinase kinase 6	Mek6-SG	0.8	StressGen KAP-MA014E
25.	MAP kinase kinase kinase 1	Mekk1-PNT	1.2	StressGen KAP-SA010
26.	MAP kinase kinase kinase 3	Mekk3 SG	0.5	StressGen KAP-MA013E
27.	Mos	Mos-III	1.0	StressGen KAP-MA004
28.	p38 MAP kinase	p38 Hog-CT	0.2	StressGen KAP-MA009E
29.	p21-activated kinase α	Pak (C-19)	0.8	SantaCruz sc-881
30.	Pim1	Pim1-T	1.4	StressGen KAP-ST004
31.	Protein kinase A (cAMP-dep. kinase)	PKA-NT	3.0	StressGen KAP-PK001
32.	Protein kinase C β	PKC-β M7 mAb	0.5	Gift from Susan Jaken[3]
33.	Protein kinase C ε	nPKC-ε (C-15)	0.5	SantaCruz sc-214
34.	Protein kinase C ζ	PKC-ζ (C-20)	0.5	SantaCruz sc-226
35.	Protein kinase G (cGMP-dep. kinase)	PKG1-CT	2.6	StressGen KAP-PK005
36.	Protein kinase B α	PKB-CT	1.8	Upstate 06-276
37.	Protein kinase B β	PKB2-PCT	2.0	CRYDSLGLLEDQRT[2]
38.	RafB	RafB-CT	3.0	StressGen KAP-MA008
39.	Ribosomal S6 kinase 1	Rsk1 (C-21)	0.7	SantaCruz sc-231
40.	Ribosomal S6 kinase 2	Rsk2-PCT	0.8	StressGen KAP-ST007
41.	S6 protein kinase	S6K-PNT	1.8	Upstate 06-321
42.	Stress-activated kinase (Jnk)	SAPKβ	1.6	StressGen KAP-SA004
43.	TGFβ-activated kinase	Tak1-CT	1.0	StressGen KAP-ST009E

Notes

(1) Upstate = Upstate Biotechnology Inc. (Lake Placid, New York, U.S.A.); StressGen = StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada); Santa Cruz Biotechnology, Inc. (Santa Cruz, California, U.S.A.).

(2) All of these antibodies are commercially available, except for Cot-PCT, Erk5-PNT, Ksr1-CT, PKC-β M7 and PKB2-PCT. For these antibodies, the amino acid sequence of the immunizing peptide is provided.

(3) All of the antibodies used were rabbit polyclonal antibodies with the exception of the PKC-β M7 mouse monoclonal antibody provided by Dr. Susan Jaken's of the W. Alton Jones Cell Science Center in Lake Placid, New York.

Gel electrophoresis and immunoblotting

The thawed cell lysates were measured for protein content using Bradford reagent (Bio-Rad) with bovine serum albumin as the reference standard. The protein concentration of the lysates was adjusted to 1 mg/ml in SDS-PAGE sample buffer (2% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol and 0.01% bromophenol blue) and boiled at 100 °C for 3 min. One mg of the cell lysate was loaded on to the stacking layer (2 mm x 4 cm x 20 cm; 4% acrylamide/0.11% bisacrylamide) of an SDS-PAGE gel. A comb was not used to create individual lanes, so that there was a single, wide lane over the width of the entire gel. The stacking gel was previously layered over a separating SDS-PAGE gel (2 mm x 12.5 cm x 20 cm; 13% acrylamide/0.086% bisacrylamide). Electrophoresis was performed at 30 mA (maximum voltage) and was continued until proteins of 25,000 Daltons had migrated to the bottom of the gel. The composition and concentrations of the other ingredients in the stacking and separate gels, and in the lower and upper chamber gel buffers were as described (Laemmli, U.K. (1970) *Nature* 227, 680-685). Proteins were then electrophoretically transferred from the gel at 300 mA (maximum voltage) for 3 h on to a nitrocellulose membrane, and the membrane was subsequently cut vertically into 1 cm wide strips. The strips were then blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the membrane with TBST, each strip was exposed to a unique mixture of different primary antibodies in TBST for 3 h with constant shaking at room temperature. The concentrations of the antibodies that were used are provided in Table 1. The strips were washed two times for 15 min with TBST and incubated with horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing the strips three times for 10 min with TBST, the strips were reassembled, and subjected to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.). Exposure of the x-ray films was for 40 sec.

Results

Figure 1 shows an example of the application of multi-kinase immunoblotting technique applied to three different rat tissues. It is evident that the patterns of kinase expressions differed markedly between the tissues. At least 45 known protein kinases were visualized on the immunoblots and clearly identified based on their predicted sizes and immunoreactivities. From the intensity of the signals for the immunoreactive kinase bands in Fig. 1, the relative expression levels of these specific protein kinases are provided in Table 2. This sort of analysis could be especially useful for tracking kinases as a function of development, long term mitogen, stress or drug stimulation, and disease progression.

Table 2. Relative expression of known protein kinases in rat tissues.

	Kinase	Relative Expression Brain	Heart	Skeletal muscle
1.	Calmodulin-dependent kinase kinase	Moderate	Low	Low
2.	Calmodulin-dependent kinase 4	Moderate	None	None
3.	Cyclin-dependent kinase 1 (cdc2)	Low	None	None
4.	Cyclin-dependent kinase 2	None	Low	None
5.	Cyclin-dependent kinase 5	Moderate	None	None
6.	Cyclin-dependent kinase 6	Low	Moderate	Moderate
7.	Cyclin-dependent kinase 7	Moderate	Low	Low
8.	Cyclin-dependent kinase 8	Low	Low	Low
9.	Casein kinase 1 α	Low	Low	Low
10.	Casein kinase 1 ε	Moderate	Low	Low
11.	Casein kinase 2 α	High	High	High
12.	Cot (Tpl2)	Low	High	Low
13.	Erk1 MAP kinase	High	High	High
14.	Erk2 MAP kinase	High	High	High
15.	Erk5 MAP kinase (Bmk)	Moderate	None	None
16.	Glycogen synthase kinase β	High	High	Moderate
17.	Integrin linked kinase 1	Moderate	Moderate	Moderate
18.	Kkialre Cdk-like kinase	None	Moderate	High
19.	Kinase-suppressor of Ras	Low	None	None
20.	MAP kinase-activated kinase 2	High	Low	None
21.	MAP kinase kinase 1 (Mek1)	High	Low	Low
22.	MAP kinase kinase 3 (Mek3)	Low	Low	Low
23.	MAP kinase kinase 4 (Mek4)	High	Low	Low
24.	MAP kinase kinase 5 (Mek5)	High	Moderate	Low
25.	MAP kinase kinase 6 (Mek6)	High	High	High
26.	MAP kinase kinase kinase 1 (Mekk1)	Low	Moderate	Low
27.	MAP kinase kinase kinase 3 (Mekk3)	Low	Low	Low
28.	Mos	None	Low	Moderate
29.	p38 α MAP kinase	Moderate	High	Moderate
30.	p21-activated kinase α	High	Low	Low
31.	Pim1	Moderate	High	Low
32.	Protein kinase A (cAMP-dep. kinase)	Moderate	High	High
33.	Protein kinase B α (Akt1)	High	Low	Low
34.	Protein kinase B β (Akt2)	High	Low	Low
35.	Protein kinase C β	High	Low	Low
36.	Protein kinase C ε	High	High	High
37.	Protein kinase C ζ	High	High	High
38.	Protein kinase G (cGMP-dep. kinase)	Low	High	High
39.	RafB	High	Low	None
40.	Ribosomal S6 kinase 1	High	High	Moderate
41.	Ribosomal S6 kinase 2	Low	Low	High
42.	S6 protein kinase	High	Low	High
43.	Stress-activated kinase β p46 (Jnk)	High	Low	Low
44.	Stress-activated kinase β p54 (Jnk)	High	Moderate	moderate
45.	TGFβ-activated kinase	Low	Low	Low

Example 2

In this example, the kinase multi-blot analysis is used to probe for the presence and activation states of over 45 different protein kinases in soluble extracts prepared from the human Ramos B cell line that have been treated anti-IgM antibody in order to stimulate these cells through a B cell antigen receptor. The results demonstate the band shifting of several protein kinases as a consequence of their increased phosphorylation in response to B cell antigen receptor stimulation.

Materials

Affinity-purified rabbit polyclonal or monoclonal antibodies and immunizing peptides used to raise these antibodies are listed in Table 1. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was obtained from Calbiochem. Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were obtained from Amersham Pharmacia Biotech, Inc. Other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

Preparation of cell extracts

The human Ramos B cell line (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle medium containing 10% heat inactivated fetal bovine serum and 2 mM glutamine at 37°C in a 5% CO_2/air mixture. For each experimental analysis, 2×10^7 cells were seeded in a 150 mm culture dish containing 20 ml of medium. Twelve hours prior to cell stimulation, the cells were cultured in the above media in the absence of serum, and then were incubated for 5 min with anti-IgM antibody. Subsequently the cells were lysed in 2 ml of ice-cold buffer that contained 20 mM MOPS, pH 7.2, 5 mM EGTA, 1% (w/v) Nonidet P-40, 1 mM dithiothreitol, 75 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride by sonication with a Branson Probe Sonicator at 4°C with 3 x 30 s bursts. The homogenates were

ultracentrifuged at 100,000 rpm (240,000 x g) for 15 min in a Beckman TLA-100.2 ultracentrifuge at 4 °C. The supernatants were immediately frozen at -70 °C until subsequent analysis.

Gel electrophoresis and immunoblotting

The thawed cell lysates were measured for protein content using Bradford reagent (Bio-Rad) with bovine serum albumin as the reference standard. The protein concentration of the lysates was adjusted to 1 mg/ml in SDS-PAGE sample buffer (2% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol and 0.01% bromophenol blue) and boiled at 100 °C for 3 min. One mg of the cell lysate was loaded on to the stacking layer (2 mm x 4 cm x 20 cm; 4% acrylamide/0.11% bisacrylamide) of an SDS-PAGE gel. A twenty lane comb was used, and the extracts from untreated (control) and anti-IgM-treated (experimental) cells were deposited into adjacent lanes. Molecular mass markers (glycogen phosphorylase, bovine serum albumin, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase) were applied to the first and last lanes of the gel. The stacking gel was previously layered over a separating SDS-PAGE gel (2 mm x 12.5 cm x 20 cm; 13% acrylamide/0.086% bisacrylamide). Electrophoresis was performed at 30 mA (maximum voltage) and was continued until proteins of 25,000 Daltons had migrated to the bottom of the gel. The composition and concentrations of the other ingredients in the stacking and separate gels, and in the lower and upper chamber gel buffers were as described (Laemmli, U.K. (1970) *Nature* 227, 680-685). Proteins were then electrophoretically transferred from the gel at 300 mA (maximum voltage) for 3 h on to a nitrocellulose membrane, and the membrane was subsequently cut vertically into strips that contained one lane of the control and one lane of the experimental samples. The strips were then blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the membrane with TBST, each strip was exposed to a unique mixture of different primary antibodies in TBST for 3 h with constant shaking at room temperature. The concentrations of the antibodies that were used are provided in Table 1. The strips were washed two times for 15 min with TBST and incubated with horse radish peroxidase-conjugated secondary

antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing the strips three times for 10 min with TBST, the strips were reassembled, and subjected to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.). Exposure of the x-ray films was for 40 sec.

Results

In the human Ramos B cell line, many of the tested protein kinases were not detected at measurable levels by the kinase multi-blot analysis. Several of the protein kinases present in the Ramos cells were observed to undergo band shifts to high apparent molecular mass species following short term exposure for 5 min to the anti-IgM antibody. These kinases are evident in Fig. 2. This band shifting generally correlated with the phosphorylation and activation of these kinases. Table 3 summarizes the findings from this experiment with respect to the expression level and band shifting of the 45 known protein kinases that were examined. An unidentified 52-kDa immunoreactive protein (p52) in Lanes 1 and 2 that band shifted upon anti-IgM antibody treatment may correspond to a novel protein kinase within the MAP kinase family based on immunoreactivity.

Table 3. Protein kinase expression and band shifting in anti-IgM antibody-treated Ramos cells.

	Kinase	Expression Level	Band Shifted
1.	Calmodulin-dependent kinase kinase	None	
2.	Calmodulin-dependent kinase 4	None	
3.	Cyclin-dependent kinase 1 (cdc2)	None	
4.	Cyclin-dependent kinase 2	None	
5.	Cyclin-dependent kinase 5	None	
6.	Cyclin-dependent kinase 6	None	
7.	Cyclin-dependent kinase 7	None	
8.	Cyclin-dependent kinase 8	None	
9.	Casein kinase 1 α	High	No
10.	Casein kinase 1 ε	None	
11.	Casein kinase 2 α	High	No
12.	Cot (Tpl2)	High	No
13.	Erk1 MAP kinase	High	Yes
14.	Erk2 MAP kinase	High	?
15.	Erk5 MAP kinase (Bmk)	None	
16.	Glycogen synthase kinase β	None	
17.	Integrin linked kinase 1	None	
18.	Kkialre Cdk-like kinase	None	
19.	Kinase-suppressor of Ras	None	
20.	MAP kinase-activated kinase 2	High	No
21.	MAP kinase kinase 1 (Mek1)	High	No
22.	MAP kinase kinase 3 (Mek3)	None	
23.	MAP kinase kinase 4 (Mek4)	None	
24.	MAP kinase kinase 5 (Mek5)	Low	No
25.	MAP kinase kinase 6 (Mek6)	Moderate	No
26.	MAP kinase kinase kinase 1 (Mekk1)	None	
27.	MAP kinase kinase kinase 3 (Mekk3)	None	
28.	Mos	None	
29.	p38 α MAP kinase	Moderate	No
30.	p21-activated kinase α	None	
31.	Pim1	None	
32.	Protein kinase A (cAMP-dep. kinase)	None	
33.	Protein kinase B α (Akt1)	None	
34.	Protein kinase B β (Akt2)	High	Yes
35.	Protein kinase C β	High	Yes
36.	Protein kinase C ε	High	Yes
37.	Protein kinase C ζ	None	
38.	Protein kinase G (cGMP-dep. kinase)	None	
39.	RafB	None	
40.	Ribosomal S6 kinase 1	High	Yes
41.	Ribosomal S6 kinase 2	None	
42.	S6 protein kinase	Moderate	Yes
43.	Stress-activated kinase β p46 (Jnk)	None	
44.	Stress-activated kinase β p54 (Jnk)	Moderate	No
45.	TGFβ-activated kinase	High	No

Example 3

In this example, the kinase multi-blot analysis is used to probe for the effect of various protein kinase inhibitors on the ability of hepatocyte growth factor (HGF) to activate protein kinases in the Erk1/Erk2, p38 MAP kinase and S6 kinase pathways. This example demonstrates that unique kinase band shift patterns may be produced by different drugs. This could be exploited to determine the mechanisms of action of known drugs and the identification of unknown targets of new drugs.

Materials

The protein kinase inhibitors PD98059 (for Mek1), LY294002 (for phosphatidylinositol 3-kinase), SB203580 (for p38 MAP kinase) and rapamycin (for mTor/FRAP) were obtained from ProMega Corporation (Madison, WI). Affinity-purified rabbit polyclonal or monoclonal antibodies and immunizing peptides used to raise these antibodies are listed in Table 1. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was obtained from Calbiochem. Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were obtained from Amersham-Pharmacia Biotech, Inc. Other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

Preparation of cell extracts

Primary cultures of human ovarian surface epithelial cells were cultured in Dulbecco's modified Eagle medium containing 10% heat inactivated fetal bovine serum and 2 mM glutamine at 37°C in a 5% CO_2/air mixture. For each experimental analysis, 2 x 10^7 cells were seeded in a 150 mm culture dish containing 20 ml of medium. Twelve hours prior to cell stimulation, the cells were cultured in the above media in the absence of serum, and then were incubated at 37°C in the absence or presence of either 50 μM PD98059, 50 μM LY294002, 10 μM SB203580 or 20 nM rapamycin for 30 min. The cells were subsequently incubated for another 10 min with 20 ng/ml of HGF prior to their lysis at 4°C in 2 ml of buffer that contained 20 mM MOPS, pH 7.2, 5 mM EGTA, 1% (w/v)

Nonidet P-40, 1 mM dithiothreitol, 75 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. The cells were sonicated with a Branson Probe Sonicator at 4°C with 3 x 30 s bursts. The homogenates were ultracentrifuged at 100,000 rpm (240,000 x g) for 15 min in a Beckman TLA-100.2 ultracentrifuge at 4 °C. The supernatants were immediately frozen at -70 °C until subsequent analysis.

Gel electrophoresis and immunoblotting

The thawed cell lysates were measured for protein content using Bradford reagent (Bio-Rad) with bovine serum albumin as the reference standard. The protein concentration of the lysates was adjusted to 1 mg/ml in SDS-PAGE sample buffer (2% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol and 0.01% bromophenol blue) and boiled at 100 °C for 3 min. One mg of the cell lysate was loaded on to the stacking layer (2 mm x 4 cm x 20 cm; 4% acrylamide/0.11% bisacrylamide) of an SDS-PAGE gel. A twenty lane comb was used, and the extracts from untreated (control; Lane 1), HGF-treated (Lane 2), PD98059- and HGF-treated (Lane 3), LY294002- and HGF-treated (Lane 4), SB203580- and HGF-treated (Lane 5), and rapamycin- and HGF-treated (Lane 6) cells were deposited into adjacent lanes. Molecular mass markers (glycogen phosphorylase, bovine serum albumin, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase, trypsinogen) were applied to the first and last lanes of the gel. The stacking gel was previously layered over a separating SDS-PAGE gel (2 mm x 12.5 cm x 20 cm; 13% acrylamide/0.086% bisacrylamide). Electrophoresis was performed at 30 mA (maximum voltage) and was continued until proteins of 25,000 Daltons had migrated to the bottom of the gel. The composition and concentrations of the other ingredients in the stacking and separate gels, and in the lower and upper chamber gel buffers were as described (Laemmli, U.K. (1970) *Nature* 227, 680-685). Proteins were then electrophoretically transferred from the gel at 300 mA (maximum voltage) for 3 h on to a nitrocellulose membrane, and the membrane was subsequently cut vertically into strips that contained six lanes of the control and various HGF and inhibitor-treated cells. The strips were then blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the

membrane with TBST, each strip was exposed to a unique mixture of different primary antibodies in TBST for 3 h with constant shaking at room temperature. The concentrations of the antibodies that were used are provided in Table 1. The strips were washed two times for 15 min with TBST and incubated with horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing the strips three times for 10 min with TBST, they were subjected to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.). Exposure of the x-ray films was for 40 sec.

Results

Many different protein kinases were clearly detected in the human ovarian surface epithelial (OSE) cells. For the purposes of this example, only the band shifted states of six protein kinases are presented in Figure 3. These kinases are the extracellular regulated kinases Erk1 (Panel A) and Erk2 (Panel A) isoforms, p38 Hog MAP kinase (Panel B), protein kinase B alpha (PKB1, Panel C) and beta (PKB2, Panel D) isoforms, and p70 S6 kinase (S6K). A schematic of the signalling pathways within which these kinases participate is present on the right in Figure 3. On the one hand, HGF is known to stimulate the enzyme activities of the Erk1 and Erk2 MAP kinases as well as p70 S6K. On the other hand, there are no reports of activation of p38 MAP kinase by HGF. In concordance, HGF treatment of the OSE cells produced reduced mobilities of Erk1, PKB1, PKB2 and S6K, consistent with their phosphorylation and activation. A band shift in Erk2 was not evident due to comigration of activated Erk2 with the inactive form of Erk1. There was no change in the mobility in p38 MAP kinase. Consistent with the known action of PD98059, the Mek1 inhibitor prevented the HGF-induced band shift in Erk1, but not of PKB1 and PKB2. There was some reduction of the HGF-induced S6K band shift, possibly because some of the phosphorylation of S6K may be catalyzed by Erk1 and Erk2. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 caused a slight reduction in HGF-induced Erk1 band shifting, in part because the protein kinase C zeta (PKCζ) isoform is normally activated by the lipid products of the PI3K reaction, and PKCζ causes the activation of Mek1. The inhibition of PI3K by LY294002 completely blocked the

HGF-induced band shifting of PKB1, PK2 and S6K, consistent with their actions distal to PI3K. Furthermore, the LY294002 produced band shifts in these kinases to forms of lower molecular mass than were detected in the untreated cells. These findings indicated that there was an intermediate state of activation of these kinases in the control cells in the absence of HGF. The p38 MAP kinase inhibitor SB203580 had no discernable effect on any of the kinases in the absence or presence of HGF. The mTOR/Frap inhibitor rapamycin only reduced the band shift in S6K, which is expected since this protein kinase appears to lie upstream of only the S6K and none of the other protein kinase that were tracked.

One application of the multi-kinase analysis is for drug profiling. It is evident in Example 3, that the different protein kinase inhibitors generated distinct changes in the 6 protein kinases that were tracked. Potentially several hundred protein kinases can be monitored for the specific effects of selected drugs. The short term actions of the drug on the basal, mitogen-stimulated and stress-stimulated phosphorylation states of the various protein kinases can be assessed. Distinct sets of band shifts should be produced by different drugs. These patterns can be interpreted to deduce the mechanisms of action of these drugs. By matching the patterns of kinase alterations induced by known drugs, it would be possible to determine targets of unknown drugs. If two drugs generate exactly the same patterns of kinase changes, then they should have the same cellular target.

Example 4

In this example, the kinase multi-blot analysis is used to probe for changes in the expression of protein kinases in extracts from human breast tumours compared to patient-matched "normal" breast tissue. The method allows for the detection of known protein kinases and other proteins that are increased in samples from diseased tissues or cells. These proteins can serve as markers of disease progression and possibly targets for therapeutic intervention.

Materials

Affinity-purified rabbit polyclonal or monoclonal antibodies and immunizing peptides used to raise these antibodies are listed in Table 1. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was obtained from Calbiochem. Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were obtained from Amersham Pharmacia Biotech, Inc. Other reagents were from Sigma-Aldrich, unless otherwise stated.

Tissue procurement and homogenization

Breast tumours from patients and their adjacent control samples were obtained through the Pathology Department at Vancouver General Hospital. The samples were immediately placed in liquid nitrogen until analysis. Homogenization was carried out by placing the tissue in 5 ml of buffer containing 20 mM MOPS, 50 mM β-glycerophosphate, 1% NP40, 50 mM sodium fluoride, 1 mM Na_3VO_4, 5 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine and 1 mM phenylmethanesulphonyl fluoride, 10 µg/ml of leupeptin and aprotinin, and applying three, 20 second bursts of a Brinkman Polytron at a setting of 10,000 rpm whilst on ice. The samples were then subjected to centrifugation at 150,000 x g for 30 min and the supernatant fractions were stored at -70°C until used.

Gel electrophoresis and immunoblotting

The thawed cell lysates were measured for protein content using Bradford reagent (Bio-Rad) with bovine serum albumin as the reference standard. The protein concentration of the lysates was adjusted to 1 mg/ml in SDS-PAGE sample buffer (2% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1 M β-mercaptoethanol and 0.01% bromophenol blue) and boiled at 100 °C for 3 min. One mg of the cell lysate was loaded on to the stacking layer (2 mm x 4 cm x 20 cm; 4% acrylamide/0.11% bisacrylamide) of an SDS-PAGE gel. A twenty lane comb was used, and the extracts from patient-matched normal (control) and tumour breast tissue biopsy extracts were deposited into adjacent lanes. The

stacking gel was previously layered over a separating SDS-PAGE gel (2 mm x 12.5 cm x 20 cm; 13% acrylamide/0.086% bisacrylamide). Electrophoresis was performed at 30 mA (maximum voltage) and was continued until proteins of 25,000 Daltons had migrated to the bottom of the gel. The composition and concentrations of the other ingredients in the stacking and separate gels, and in the lower and upper chamber gel buffers were as described (Laemmli, U.K. (1970) *Nature* 227, 680-685). Proteins were then electrophoretically transferred from the gel at 300 mA (maximum voltage) for 3 h on to a nitrocellulose membrane, and the membrane was subsequently cut vertically into strips that contained one lane of the control and one lane of the experimental samples. The strips were then blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the membrane with TBST, each strip was exposed to a unique mixture of different primary antibodies in TBST for 3 h with constant shaking at room temperature. The concentrations of the antibodies that were used are provided in Table 1. The strips were washed two times for 15 min with TBST and incubated with horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing the strips three times for 10 min with TBST, they were subjected to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.). Exposure of the x-ray films was for 2 min.

Results

Many different known protein kinases were clearly detected in the human breast tumours and patient matched "normal" breast biopsy samples. Most of these kinases were unaltered in their relative expression levels. Five protein kinases that consistently exhibited increased amounts in the tumour samples are presented in set of left panels in Figure 4. These kinases were p38 Hog MAP kinase, protein kinase B-alpha (PKBα), the various isoforms of the catalytic subunit of casein kinase 2 (CK2), cGMP-dependent protein kinase (PKG) and cyclin-dependent kinase 8 (Cd8). The elevated amounts of one or more of these kinases may contribute to the neoplastic transformation of breast tissue. Alternatively, they may represent feedback responses to counteract the loss of growth

control in the breast tumours. In either event, they may serve as useful markers of cancer progression.

Many antibodies are able to cross-react with related proteins that share the epitopes that are recognized for binding by these antibodies. Since most protein kinases are evolutionarily related, there is a high probability of cross-reactivity of kinase-directed antibodies with homologous kinases. In the experiment described in this example, there were at least 12 unidentified immunoreactive proteins with kinase antibodies that were selectively detected in the tumour samples, but poorly if at all the control samples. Five of these proteins are shown in the immunoblots in the set of panels on the right side of Figure 4. It is possible to identify these proteins following their enrichment by standard purification techniques and protein microsequence analysis. Three major advantages of kinase purification with antibodies are: (1) it is unnecessary to know what will serve as a selective substrate to monitor the presence of the kinase during its purification; (2) it is not critical to preserve the enzyme activity of the kinase during the purification procedures; and (3) the position of the kinase can be clearly detected on an SDS-PAGE gel by immunoblotting for its sequencing, even if the protein is not completely pure.

Example 5

In this example, standard immunoblotting of 2D isoelectric focusing/SDS-PAGE gels of rat brain extracts was undertaken to demonstrate the limitations of the traditional approach for proteomic analysis as applied to protein kinases.

Materials

The Immobiline DryStrip Kit with immobilized IPG ampholytes was obtained from Amersham Pharmacia Biotech, Inc. The rabbit polyclonal antibodies Erk1-CT (Catalogue No. 06-182) and PKC-III (Catalogue No. KAP-PK003) were obtained from Upstate Biotechnology Inc. and StressGen Biotechnologies Corp., respectively. Goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) were obtained from Calbiochem.

Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were obtained Amersham Pharmacia Biotech, Inc. Other reagents were from Sigma-Aldrich, unless otherwise stated.

Preparation of rat brain extract

The brain from a 50-day old male Sprague-Dawley rat was rapidly excised, after induction of anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg). The brain was cut, rinsed with phosphate buffered saline at 4 °C, and re-suspended in 10 volumes of ice-cold homogenization buffer containing: 10 mM Tris, pH 7.2, 0.1 mM EGTA, 0.1 mM Na_2EDTA, 1 mM Na_3VO_4, 50 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 10% glycerol and 1% Triton X-100. This was then homogenized with a Brinkman Polytron homogenizer. The homogenates were ultracentrifuged at 100,000 rpm (240,000 x g) for 15 min in a Beckman TLA-100.2 ultracentrifuge at 4 °C. The supernatant was immediately frozen at -70 °C until subsequent analysis.

Gel electrophoresis and immunoblotting

The thawed cell lysate was measured for protein content using Bradford reagent (Bio-Rad) with bovine serum albumin as the reference standard. One mg of the soluble protein was applied to an Immobiline isoelectric focusing (IEF) DryStrip and electrophoresis was performed on Hoefer SE600 standard vertical gel system as recommended by the manufacturer (Pharmacia Biotech). The 18 cm, pH 3-10 DryStrip was previously rehydrated in 6 M urea, 2 M thiourea, 4% CHAPS, 0.02% bromophenol blue, 2% Nonidet P-40, 0.7% dithiothreitol, 10 mM Tris, 2% IPG ampholyte, 10% glycerol, and 4 mM tributyl phosphine. Following the isoelectric focusing, the IPG strip was overlaid lengthwise on to an 11% SDS-polyacrylamide gel, and electrophoresis was continued into the second dimension. In a separate lane at the left side of the same 11% SDS-PAGE gel, 200 µg of the cytosolic protein was directly loaded on to the gel prior to the electrophoresis into the second dimension. Subsequently, the resolved proteins were

then electrophoretically transferred from the 2D gel at 300 mA (maximum voltage) for 3 h on to a nitrocellulose membrane. The membrane was then blocked with 5% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the membrane with TBST, it was exposed to a mixture of 0.2 µg/ml of Erk1-CT and 1 mg/ml of the PKC-III primary antibodies in TBST for 3 h with constant shaking at room temperature. The membrane was washed two times for 15 min with TBST and incubated with horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) in TBST for 30 min. After washing the membrane three times for 10 min with TBST, it was subjected to the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.). Exposure of the x-ray film was for 5 min.

Results

The migration positions of PKCβ, Erk1 and Erk2 are shown in Figure 5. The right most of the series of spots for Erk1 and Erk2 correspond to their fully dephosphorylated states, and these proteins shifted progressively to the left with the acquisition of each phosphate group. Only one large spot was evident for PKCβ. The large black smear on the left of the 2D gel corresponds to five-fold less brain extract applied directly to the same SDS-PAGE gel than was also loaded on to the IPG Drystrip, which was then transferred into the SDS-PAGE gel. These results were consistently observed in multiple experiments, even when diverse agents were used to try to release the proteins from the IPG Drystrip. The adsorption of protein to the IPG Drystrip was especially problemmatic for less abundant cellular proteins such as protein kinases. Evidently, less than 5% of the Erk1 and Erk2 that loaded on to the IPG Drystrip actually entered the SDS-PAGE gel when electrophoresis was performed in the second dimension.

The unpredictable retention of protein kinases and their substrates by the IPG Drystrips calls into question the useful of standard 2D gel electrophoresis (eg. according to O'Farrell) for resolution of proteins to perform quantitative studies to monitor changes in protein expression and post-translational covalent modification. It is possible that the

phosphorylation state of proteins may also influence their binding to the IPG Drystrips. This would compromise on the analysis of the relative amounts of the dephosphorylated to phosphorylated species of some proteins on 2D gels. It is possible that the problems associated with protein retention on the IPG Drystrips with immobilized ampholytes might be avoided through the use of tube gels and soluble ampholytes. However, the protein loading capacity of such tube gels is markedly lower. This could still result in insufficient protein on the 2D gel for detection of protein kinases by silver staining or immunostaining.

An attempt was made to detect more than twenty different protein kinases by immunoblotting 2D gels of rat brain lysates with the antibodies described in Table 1. The immunoblots were performed with mixes composed of two to four different antibodies. With few exceptions, it was very difficult to detect any spots that corresponded to the appropriate sizes of the target proteins. The data shown in Figure 5 represents the best finding using particularily sensitive antibodies. 2D gel electrophoresis is thus insufficiently sensitive and inconsistently reproducible for the simultaneous detection of rare proteins such as protein kinases.

EXAMPLE 6

Determination of the Coexpression Quotient values for a kinase network

For simplicity, the interrelationships of only 20 different protein kinases (named A to T) are considered in 10 different model systems (named 1 to 10). In Table 4, the densiometric intensity values for these protein kinases are listed. These would be determined under conditions where the signal intensity by densiometric analysis is linear with the exposure time of the x-ray film by ECL detection.

Table 4. Densiometric values of twenty protein kinases in ten different model systems.

System / Kinase	1	2	3	4	5	6	7	8	9	10
A	85	180	142.5	160	100	95	360	255	200	90
B	22.5	42.5	37.5	50	25	25	85	75	47.5	25
C	50	95	63.75	100	47.5	50	200	127.5	100	42.5
D	190	400	300	400	200	200	800	600	300	200
E	67.5	127.5	112.5	142.5	75	75	255	11.25	0	7.5
F	25	50	37.5	50	25	25	100	0	2.5	0
G	112.5	212.5	187.5	250	12.5	0	25	0	0	0
H	50	100	67.5	90	0	0	30	0	5	2.5
I	0	0	225	255	150	150	540	450	0	15
J	0	37.5	159.4	250	125	125	475	318.8	0	0
K	3.75	0	112.5	142.5	63.8	75	270	225	0	0
L	0	0	75	100	45	50	200	142.5	5	5
M	0	0	0	0	100	85	400	300	20	0
N	5	5	0	0	25	25	100	75	0	0
O	0	0	9.4	12.5	106.3	125	475	318.75	37.5	12.5
P	0	0	0	0	150	142.5	600	450	0	0
Q	50	85	11.25	0	0	0	0	15	100	42.5
R	100	200	0	10	0	10	60	0	190	100
S	21.25	50	1.9	0	2.5	0	0	0	42.5	22.5
T	125	225	0	0	0	0	50	0	250	118.75
Erk1	50	100	75	100	50	50	200	150	100	50

These densiometric values from different model systems have to be normalized by expressing them relative to the densiometric intensity determined for Erk1 in rat brain with Erk1-CT antibody. This is provided in the bottom row of Table 4. This permits the estimation of the R.O.D. values shown in Table 5.

Table 5. Relative Optical Density values of twenty protein kinases in ten different model systems.

System / Kinase	1	2	3	4	5	6	7	8	9	10	Max value
A	170	180	190	160	200	190	180	170	200	180	200
B	45	42.5	50	50	50	50	42.5	50	47.5	50	50
C	100	95	85	100	95	100	100	85	100	85	100
D	380	400	400	400	400	400	400	400	300	400	400
E	135	127.5	150	142.5	150	150	127.5	7.5	0	15	150
F	50	50	50	50	50	50	50	0	2.5	0	50
G	225	212.5	250	250	25	0	12.5	0	0	0	250
H	100	100	90	90	0	0	15	0	5	5	100
I	0	0	300	255	300	300	270	300	0	30	300
J	0	37.5	212.5	250	250	250	237.5	212.5	0	0	250
K	7.5	0	150	142.5	127.5	150	135	150	0	0	150
L	0	0	100	100	90	100	100	95	5	10	100
M	0	0	0	0	200	170	200	200	20	0	200
N	10	5	0	0	50	50	50	50	0	0	50
O	0	0	12.5	12.5	212.5	250	237.5	212.5	37.5	25	250
P	0	0	0	0	300	285	300	300	0	0	300
Q	100	85	15	0	0	0	0	10	100	85	100
R	200	200	0	10	0	20	30	0	190	200	200
S	42.5	50	2.5	0	5	0	0	0	42.5	45	50
T	250	225	0	0	0	0	25	0	250	237.5	250

These R.O.D. values for a given protein kinase have to be compared in the different model systems by expressing them relative to maximal R.O.D. value determined for that kinase. This permits the estimation of the R.E. values shown in Table 6.

Table 6 Relative Expression values of twenty protein kinases in ten different model systems.

System	1	2	3	4	5	6	7	8	9	10
Kinase										
A	85	90	95	80	100	95	90	85	100	90
B	90	85	100	100	100	100	85	100	95	100
C	100	95	85	100	95	100	100	85	100	85
D	95	100	100	100	100	100	100	100	75	100
E	90	85	100	95	100	100	85	5	0	10
F	100	100	100	100	100	100	100	0	5	0
G	90	85	100	100	10	0	5	0	0	0
H	100	100	90	90	0	0	15	0	5	5
I	0	0	100	85	100	100	90	100	0	10
J	0	15	85	100	100	100	95	85	0	0
K	5	0	100	95	85	100	90	100	0	0
L	0	0	100	100	90	100	100	95	5	10
M	0	0	0	0	100	85	100	100	10	0
N	10	5	0	0	100	100	100	100	0	0
O	0	0	5	5	85	100	95	85	15	10
P	0	0	0	0	100	95	100	100	0	0
Q	100	85	15	0	0	0	0	10	100	85
R	100	100	0	5	0	10	15	0	95	100
S	85	100	5	0	10	0	0	0	85	90
T	100	90	0	0	0	0	10	0	100	95

In each model system, the difference in the R.E. values of the two proteins would next be determined. These differences would be summed and then divided by the number (N=10) of model systems in which the two proteins were compared to derive the C.E.Q. value. For example, Kinase A and Kinase H are compared as follows:

System	1	2	3	4	5	6	7	8	9	10	
Kinase											
A	85	90	95	80	100	95	90	85	100	90	
H	100	100	90	90	0	0	15	0	5	5	
Difference	15 +	10 +	5 +	10 +	100 +	95 +	75 +	85 +	95 +	85	= 575

C.E.Q. = 575 / 10 = 57.5

Table 7 Coexpression Quotient values of twenty protein kinases.

Kinase	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A	0	7.5	7.5	11	28.5	33	58	57.5	38	39	41.5	41	56.5	55.5	52	56.5	54.5	55.5	55.5	55.5
B		0	9	5.5	9.5	33	56.5	60	38	39.5	39	38.5	58.5	57	57.5	59	58	58	61	70
C			0	8.5	31	29	57.5	56	43	37.5	43	39.5	59	57	53.5	59	55	55.5	59	57
D				0	29.5	27.5	58	57.5	38.5	39	39.5	37	57	55.5	57	57.5	62.5	59.5	61.5	63.5
E					0	6.5	29	32.5	28.5	28	29.5	30	51.5	47.5	48	49.5	65.5	66.5	65.5	67.5
F						0	31.5	31	34	29.5	33	31.5	52	49	51.5	51	69	66	67	69
G							0	7.5	57.5	54	53	56	75.5	74.5	74	75.5	40.5	44.5	39.5	42.5
H								0	60	57.5	58	59.5	75.5	76	73.5	77	38	37	38	38
I									0	7.5	3	5.5	22	22	22.5	21	91	93	91	94
J										0	7.5	7	24.5	22.5	23	22	89.5	91.5	89.5	92.5
K											0	4.5	24.5	23	23.5	23	91	92.5	91	94
L												0	24.5	24.5	22	23.5	91.5	93.5	91.5	94.5
M													0	4	7.5	2	75	75	73	75
N														0	8.5	2	76	76	74	76
O															0	7.5	71.5	71.5	69.5	72.5
P																0	77	77	75	77
Q																	0	9	8	5
R																		0	8	4
S																			0	7
T																				0

By comparison of the C.E.Q. values for the kinase pairs, the following protein kinases appear to be associated with each other. The C.E.Q. score is taken to be very significant if it is less than 15 in the following example from Table 7.

A+B, A+C, A+D
B+C, B+D, B+E
C+D
E+F
G+H
I+K, I+L, I+J
J+K, J+L
K+L
M+N, M+O, M+P
N+O, N+P
O+P
Q+R, Q+S, Q+T
R+S, R+T
S+T

Each of these kinase pairs can be further grouped into a cluster based on both kinases in each pair being present in other pairs. The clusters would, therefore, be:

1. A+B, A+C, A+D, B+C, B+D, C+D
2. J+K, J+L, K+L, I+J, I+K, I+L
3. M+N, M+O, M+P, N+O, N+P, O+P
4. Q+R, Q+S, Q+T, R+S, R+T, S+T

This implies that:

1. Kinases A, B, C and D are commonly coexpressed and may operate together in a pathway.
2. Kinases I, J, K and L are commonly coexpressed and may operate together in a pathway.
3. Kinases M, N, O and P are commonly coexpressed and may operate together in a pathway.
4. Kinases Q, R, S and T are commonly coexpressed and may operate together in a pathway.
5. Kinases E and F are commonly coexpressed and may operate together in a pathway.
6. Kinases G and H are commonly coexpressed and may operate together in a pathway.

This analysis of the C.E.Q. values of the kinase pairs can indicate players in a common signalling pathway. However, it is not reliable for revealing the specific, direct interactions, nor does it provide the order in which these kinases may be positioned within a signalling pathway.

Analysis of post-translational coregulation of protein kinases to establish interrelationships

Protein phosphorylation is the major form of post-translation regulation of proteins in eukaryotic cells, and this reaction is carried out by protein kinases. Often when protein kinases are phosphorylated and activated, they display reduced migration during SDS-PAGE, a phenomena described as "band shifting up." The reduced mobility of phosphorylated proteins can be approximately 1 to 5 kDa on SDS-PAGE gels. It has been observed that many proteins that band shift together following Western blotting analysis of an extract prepared from animals or cells that have been treated with one or more stimuli. For example, phorbol ester tumour promoter treatment of a wide range of cells leads to upward band shifts in the protein kinases Erk2 and Rsk2. Erk2, in fact, phosphorylates and activates Rsk2, and Erk2 is phosphorylated and activated by another protein kinase, i.e. Mek1. If subsets of protein kinases exhibit a high degree of correlation with each other with respect to their band shifting by Western blotting analysis of a large number of different stimuli, then there is a high probability that they operate within common signalling pathways. Furthermore, the higher the correlation coefficient of the mobility behaviour of two protein kinases (or other proteins) on an SDS-PAGE gel, the closer that they may reside in a pathway. The greater the number of different experimental model systems under examination, the more definitive the statements that can be made about the specific linkages between protein kinases.

While there is a high probability that functionally distinct protein kinases may be expressed at any given time in the life of a cell, there is a much lower chance that that they would all be turned on or off by phosphorylation at the same time. Therefore, correlation of the phosphorylation (as reflected by band shifts) states of different protein kinases has a better prospect of uncovering direct and indirect connections between protein kinases in common signalling systems.

As an alternative to the detection of phosphorylation of a specific protein kinase or its substrate by band shifting on Western blots, it is also possible to monitor the state of phosphorylation of proteins through the use of phosphorylation site-specific antibodies. Such antibodies are commercially available, and can be generated in mice and rabbits against peptides based on phosphorylation site sequences that have been synthesized to include the phosphate or an analogue. These antibodies are phosphorylation-site specific, because they have been

depleted of immunoreactivity towards phosphorylation-deficient peptides that feature the same amino acid sequence, but which are lacking the phosphate moiety.

The coregulation quotient (C.R.Q.) is a measure of the how closely two protein kinases are similarly controlled, and therefore likely to operate in a common signalling pathway. A C.R.Q. of 1 means that both protein kinases are affected in the same way with respect to their phosphorylation states of two protein kinases. The C.R.Q.values of a group of protein kinases could be determined as follows. If a kinase (or another protein) is in the phosphorylated state it is designated "P", whereas if the kinase (or another protein) is in the dephosphorylated state, it is designated "D". Two kinases (or other proteins) are compared in each model system, and if both are phosphorylated, then they are given a value of 3. If both kinases are dephosphorylated, then they are assigned a value of 1. If one kinase is dephosphorylated and the other is phosphorylated, then they are assigned a value of -3. Once the values of the matches are determined, they are summed and then divided by the number (N) of model systems in which the two proteins were compared to derive the C.R.Q. value. Note if the protein kinase is not detectable (N.S. = no signal) in certain model systems, then these model systems are omitted from the calculations.

For example, Kinase A and Kinase B are compared in their phosphorylation states as follows:

TABLE 8

System	1	2	3	4	5	6	7	8	9	10	
Kinase											
A	P	D	D	D	P	P	D	D	N.S.	P	
B	P	D	D	D	P	P	P	D	N.S.	P	
Match	3 +	1 +	1 +	1 +	3 +	3 +	-3 +	1 +		3	= 13

C.R.Q. =13 / 9 = 1.44

In the above example, there is a high degree of correlation with respect to the regulation of Kinases A and B. The C.E.Q. will be more reliable as a larger number of model systems and perturbations are examined. It is expected that in most situations, the majority of the protein kinases (and other proteins) are in a dephosphorylated states. Consequently, the C.E.Q. numbers will always be much less than 1. If the C.E.Q. value calculates to a negative number, it is simply stated as 0.

Determination of the Coregulation Quotient values for a kinase network

In the model system, stimuli are used that differentially affect the phosphorylation states of the kinase. The objective of this example is to derived the C.R.Q. values for 20 different protein kinases. In Table 9, the phosphorylation states of these protein kinases are shown. For those protein kinases that were not detected, N.S. (no signal) is indicated.

Table 9. Phosphorylation states of twenty protein kinases in ten different model systems.

System / Kinase	1	2	3	4	5	6	7	8	9	10
A	P	D	D	D	D	D	P	P	D	D
B	P	D	D	D	D	D	P	P	D	D
C	P	P	D	D	D	D	P	P	P	P
D	P	P	D	D	D	D	P	P	P	P
E	D	P	D	P	D	D	D	N.S.	N.S.	N.S.
F	D	P	D	P	D	D	D	N.S.	N.S.	N.S.
G	D	P	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
H	D	P	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
I	N.S.	N.S.	P	P	P	D	D	P	N.S.	N.S.
J	N.S.	N.S.	P	P	P	D	D	P	N.S.	N.S.
K	N.S.	N.S.	P	P	P	P	P	P	N.S.	N.S.
L	N.S.	N.S.	P	P	P	P	P	P	N.S.	N.S.
M	N.S.	N.S.	N.S.	N.S.	P	P	P	D	N.S.	N.S.
N	N.S.	N.S.	N.S.	N.S.	P	P	P	D	N.S.	N.S.
O	N.S.	N.S.	N.S.	N.S.	P	P	P	D	N.S.	N.S.
P	N.S.	N.S.	N.S.	N.S.	P	P	P	D	N.S.	N.S.
Q	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P	P
R	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P	P
S	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P	P
T	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P	P

Table 10. Coregulation. values of twenty protein kinases.

Kinase	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A	0	1.6	0.4	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B		0	0.4	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C			0	2.2	0	0	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0
D				0	0	0	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0
E					0	1.57	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0
F						0	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0
G							0	1.5	0	0	0	0	0	0	0	0	0	0	0	0
H								0	0	0	0	0	0	0	0	0	0	0	0	0
I									0	2.33	1.0	1.0	0	0	0	0	0	0	0	0
J										0	1.0	1.0	0	0	0	0	0	0	0	0
K											0	3.0	1.5	1.5	1.5	1.5	0	0	0	0
L												0	1.5	1.5	1.5	1.5	0	0	0	0
M													0	2.5	2.5	2.5	0	0	0	0
N														0	2.5	2.5	0	0	0	0
O															0	2.5	0	0	0	0
P																0	0	0	0	0
Q																	0	2.0	2.0	2.0
R																		0	2.0	2.0
S																			0	2.0
T																				0

By comparison of the C.R.Q. values for the kinase pairs, the following protein kinases appear to be associated with each other. The C.E.Q. score is taken to be very significant if it is greater than 0 in the following example from Table 10.

A+B, A+C, A+D
B+C, B+D
C+D, C+G, C+H
D+G, D+H
E+F, E+G, E+H
F+G, F+H
G+H
I+J , I+K, I+L
J+K, J+L
K+L, K+M, K+N, K+O, K+P
L+M, L+N, L+O, L+P
M+N, M+O, M+P
N+O, N+P
O+P
Q+R, Q+S, Q+T
R+S, R+T
S+T

Each of these kinase pairs can be further grouped into a cluster based on both kinases in each pair being present in other pairs. The clusters would, therefore, be:

1. A+B, A+C, A+D, B+C, B+D, C+D, C+G, C+H, D+G, D+H, E+F, E+G, E+H, F+G, F+H, G+H,
2. I+J, I+K, I+L , J+K, J+L, K+L, K+M, K+N, K+O, K+P, M+N, M+O, M+P, N+O, N+P, O+P
3. Q+R, Q+S, Q+T, R+S, R+T, S+T

This implies that:

1. Kinases A, B, C, D, E, F, G and H are commonly coregulated and may operate in common pathways.
2. Kinases I, J, K, L, M, N, O and P are commonly coregulated and may operate in common pathways.
3. Kinases Q, R, S and T are commonly coregulated and may operate in common pathways.

This analysis of the C.E.Q. values of the kinase pairs can indicate players in common signalling pathways. However, it does not reveal the specific, direct interactions, nor does it provide the order in which these kinases may be positioned within a signalling pathway. It is unclear that each of the clustered protein kinases operate with all of the other kinases in the cluster. Another problem with this analysis is that it depends upon the ability of the analytical methods to distinguish between the phosphorylated and dephosphosphorylated states of proteins. While in most cases, this should be achievable by monitoring band shifting on SDS-PAGE gels or through employment of phosphosite-specific antibodies, there may be a few proteins where no changes are apparent.

Application of Linkage Coefficients between protein kinases to establish interrelationships

The coexpression quotient (C.E.Q.) and coregulation quotient (C.R.Q) for each protein kinase pair combination can yield valuable information about the possible interrelationships between them. These values can be used together to further identify interacting protein kinases and families. The linkage coefficient (L.C.) value is calculated from the C.R.Q. value divided by the C.E.Q. value for a kinase pair. In Table 11, the C.R.Q. values of the same twenty protein kinases presented in Table 10 are divided by their derived C.E.Q. values presented in Table 7.

Table 11. Linkage Coefficient values of twenty protein kinases.

KinaseA	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A 0	21.3	5.3	3.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	4.4	7.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C		0	25.9	0	0	0.9	0.9	0	0	0	0	0	0	0	0	0	0	0	0
D			0	0	0	0.9	0.9	0	0	0	0	0	0	0	0	0	0	0	0
E				0	24.2	1.7	1.5	0	0	0	0	0	0	0	0	0	0	0	0
F					0	1.6	1.6	0	0	0	0	0	0	0	0	0	0	0	0
G						0	20	0	0	0	0	0	0	0	0	0	0	0	0
H							0	0	0	0	0	0	0	0	0	0	0	0	0
I								0	31.1	33.3	18.2	0	0	0	0	0	0	0	0
J									0	13.3	14.3	0	0	0	0	0	0	0	0
K										0	66.7	6.1	6.5	6.4	6.5	0	0	0	0
L											0	6.1	6.1	6.8	6.4	0	0	0	0
M												0	62.5	33.3	125	0	0	0	0
N													0	29.4	125	0	0	0	0
O														0	33.3	0	0	0	0
P															0	0	0	0	0
Q																0	22.2	25	40
R																	0	25	50
S																		0	28.6
T																			0

The analysis of the L.C. values in Table 11 offers better information about the interrelationships between the twenty protein kinases. In Table 12 the scores of the L.C. analysis from Table 11 are listed in order of highest values first.

Table 2. Highest Linkage Coefficient values determined for twenty protein kinases.

Pair number	LC score	Pair
1.	125	M vs P
2.	125	N vs P
3.	67.7	K vs L
4.	62.5	M vs N
5.	50	S vs T
6.	40	Q vs T
7.	33.3	I vs J
8.	33.3	M vs O
9.	33.3	O vs P
10.	31.1	I vs K
11.	29.4	N vs O
12.	28.6	S vs T
13.	25.9	C vs D
14.	25	R vs S
15.	25	Q vs S
16.	24.2	E vs F
17.	22.2	Q vs R
18.	21.3	A vs B
19.	20	G vs H
20.	18.2	I vs L
21.	14.3	J vs L
22.	13.3	J vs K
23.	7.3	B vs D
24.	6.8	L vs O
25.	6.5	K vs N
26.	6.5	K vs P
27.	6.4	K vs O
28.	6.4	L vs P
29.	6.1	K vs M
30.	6.1	L vs M
31.	6.1	L vs N
32.	5.3	A vs C
33.	4.4	B vs C
34.	3.6	A vs D
35.	1.7	E vs G
36.	1.6	F vs G
37.	1.6	F vs H
38.	1.5	E vs H
39.	0.9	C vs G
40.	0.9	C vs H
41.	0.9	D vs G
42.	0.9	D vs H

These results are in keeping with the notion that the following kinases are linked into signalling pathways:

1. A, B, C, D
2. C, D, E, F, G, H
3. I, J, K, L
4. K, L, M, N, O, P
5. Q, R, S, T

Since A had a high L.C. value with B, it highly likely that they lie very close within the same signalling pathway. Likewise C and D appeared to be tightly correlated. C + D not only had a linkage with A + B, but also with E, F, G and H. This could be interpreted to mean that C and D lie downstream of A and B as well as E, F, G and H. Otherwise, an association of A + B with E, F ,G and H would have been evident.

Since E and F had a high L.C. value, they also appear to be linked. Similarly G and H are strongly associated. The weaker, but clear linkage of E+F with G+H indicates that they are found in a common pathway.

I, J, K and L exhibited high L.C. values with each other and likely operate within a common pathway. K and L, which had one of the highest L.C. scores with each other, were also associated with M, N, O and P. This could be interpreted to mean that K and L lie downstream of I and J as well as M, N, O and P. Otherwise, an association of I + J with M, N ,O and P would have been evident.

M, N, O and P exhibited high L.C. values with each other and likely operate within a common pathway.

Q, R, S and T exhibited high L.C. values with each other and likely operate within a common pathway.

Use of kinase inhibitors, antisense and gene therapy to establish interrelationships between protein kinases.

The analysis of the L.C. scores for protein kinases pairs is a powerful way of establishing potential connections between protein kinases and other proteins, which may serve as their regulators or substrates. In the preceeding example with 20 protein kinases, there were 400 possible interactions, and these were narrowed down to 42. The next major challenge is to delineate the order of the protein kinases within the putative pathways defined by the clusters of kinases with LC scores above 1. Only a limited amount of information in this regard could be provided by the LC analysis.

Some information about the order of protein kinases in pathways can be obtained in time course studies in which the phosphorylation states of protein kinases and their substrates are carefully monitored at various times immediately after the exposure of a cell to different stimuli. An upstream acting kinase should always become activated before a downstream kinase. In some cases, it may be useful to lower temperature at which cells are exposed to the stimuli in order to slow down the rate at which the kinases become sequentially activated. For example, in Table 13 show the differences in the rates phosphorylation changes in the 10 model systems presented in Table 9.

Table 13 Time course of phosphorylation states of twenty protein kinases in ten different model systems. The time points (in minutes) after the introduction of the stimulus in the ten different model systems at which 50% of the change in phosphorylation of each protein kinase occurs is shown.

System / Kinase	1	2	3	4	5	6	7	8	9	10	Average
A	P (1)	D	D	D	D	D	P (1)	P (2)	D	D	1.3
B	P (5)	D	D	D	D	D	P (4)	P (6)	D	D	5.0
C	P (10)	P (11)	D	D	D	D	P (9)	P (11)	P (11)	P (10)	10.3
D	P (15)	P (17)	D	D	D	D	P (16)	P (20)	P (19)	P (18)	17.5
E	D	P (2)	D	P (2)	D	D	D	N.S.	N.S.	N.S.	2.0
F	D	P (5)	D	P (6)	D	D	D	N.S.	N.S.	N.S.	5.5
G	D	P (10)	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	10
H	D	P (16)	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	16
I	N.S.	N.S.	P (2)	P (2)	P (1)	D	D	P (2)	N.S.	N.S.	1.8
J	N.S.	N.S.	P (5)	P (6)	P (5)	D	D	P (4)	N.S.	N.S.	5.0
K	N.S.	N.S.	P (9)	P (10)	P (11)	P (9)	P (10)	P (8)	N.S.	N.S.	9.5
L	N.S.	N.S.	P (17)	P (18)	P (17)	P (17)	P (19)	P (16)	N.S.	N.S.	17.3
M	N.S.	N.S.	N.S.	N.S.	P (1)	P (2)	P (1)	D	N.S.	N.S.	1.3
N	N.S.	N.S.	N.S.	N.S.	P (3)	P (5)	P (4)	D	N.S.	N.S.	4.0
O	N.S.	N.S.	N.S.	N.S.	P (12)	P (11)	P (12)	D	N.S.	N.S.	11.7
P	N.S.	N.S.	N.S.	N.S.	P (21)	P (20)	P (19)	D	N.S.	N.S.	20
Q	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P (1)	P (2)	1.5
R	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P (5)	P (6)	5.5
S	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P (10)	P (11)	10.5
T	D	D	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	P (19)	P (20)	19.5

From the data in Table 13 it can be seen that the various protein kinases became phosphorylated in different orders. By taking the average time for the 50% of maximal phosphorylation change in each kinase, kinases that operate more proximal and more distal to the earliest signalling events after introduction of a stimulus to a model system can be clearly distinguished as shown in the last column of Table 13. Therefore, the temporal order of phosphorylation of the twenty kinases can be stated as A, E, I, M & Q before B, F, J, N & R before C, G, K, O & S before D, H, L, P and T.

When this information is combined with the findings of Table 12, it can be concluded that the following pathways are in operation:

1. A→B→C→D
2. E→F→G→H
   ↓
   C→D
3. I→J→K→L
4. M→N→O→P
   ↓
   K→L
5. Q→R→S→T

Further data regarding the positioning of protein kinases within networks can be garnered through the abolition of the function of specific protein kinases. Protein kinases that lie downstream of the targeted kinases will also fail to be activated. The loss function of a kinase in a pathway may be achieved by several means. Pharmacological intervention with a specific, potent and cell permeable inhibitor is the preferred way to block signalling downstream of a protein kinase. Examples of such inhibitors are UO-126 and PD98059 for the MAP kinase kinase Mek1, SB203580 for p38/Hog MAP kinase, and rapamycin for FRAP/mTOR. As a consequence of high throughput screening for protein kinases inhibitors by many biotech and pharmaceutical companies, there will be many such inhibitors available in the near future to exploit as tools for dissecting the architecture of kinase networks.

Another method to eliminate the function of a protein kinases (or any other protein) is to use antisense to prevent the translation of mRNA for the target kinase. This approach requires more time (e.g. 24 h) in order for the antisense to exert its effect. As a consequence, there could be changes in kinase signalling that are long term responses to the loss of the function of the targeted kinase. For example, there might be up regulation of the protein levels of the other protein kinases that participate in signalling with the targeted kinase to compensate. Ideally with the lack of function of a targeted kinase, there would also be the loss of phosphorylation and activation of kinases and other proteins that operate downstream.

The expression of dominant negative versions of a target kinase in a cell is another way in which it may be inhibited. The critical conserved lysine residue in the subdomain II region of the catalytic domain of protein-serine/threonine kinases and proein-tyrosine kinases is commonly targeted for site-directed mutagenesis to generate dominant-negative kinases. The dominant-negative kinase inhibits the target kinase by interacting with its activators and substrates in non-productive complexes. The dominant-negative approach also has disadvantages in that the successful stable or transient expression of dominant-negative proteins is non-trivial, and complicated phenotypes can be produced if the dominant-negative proein also interacts with inhibitors of the target kinase.

A genetic approach in which the gene for the target protein kinase is disrupted can also be exploited to remove its function. This could be through the use of trangenic animals or cells where the gene for target kinase has been deleted or disrupted so that it is not transcribed into mRNA. Again, major disadvantages associated with gene-knockout approaches is the difficulty inherent in this technology, the potential lethality of the loss of function of the targeted gene and the widespread compensatory changes that may be produced inside a cell as consequence.

Determination of direct interactions between protein kinases, their activators and targets

All of the preceding methods provide important clues about the clustering and ordering of protein kinases within pathways. The previous methods permit a narrowing down of the candidates that are likely to interact with each other. Several other strategies exist by which the direct interaction of proteins can be established.

One of the advantages of having specific antibodies that can distinguish between different protein kinases is that they can also be deployed for coimmunoprecipitation studies. If two kinases interact, provided that the immunoprecipitating antibody does not disrupt the complex

formation, then both should be coimmunoprecipitated. This may occur only when the upstream kinase is in the active or inactive conformation.

It is possible to detect novel protein kinases and other substrates that may act downstream of a target kinase in coimmunoprecipitates by *in vitro* phosphorylation studies. This can be achieved by incubation of [γ-^{32}P]ATP with immunoprecipitates obtained with an antibody against the target kinase and lysates from cells where that kinase is highly expressed and active. Following protein microsequence analysis or mass spectrometry analysis of proteolytically (e.g. with trypsin) or chemically (e.g. with cyanogen bromide) cleaved fragments of proteins that become radiolabelled through phosphorylation in the immunoprecipitates, it is feasible to identify associated proteins.

Detection of protein kinase interactions by multi-kinase immunoblotting

The multi-kinase immunoblotting strategy in combination with the use of immunoprecipitation with specific protein kinase antibodies or employment of protein kinases as ligands for affinity chromatography is a powerful approach for establishing protein-protein interactions. Many protein kinases are routinely expressed as glutathione S-transferase (GST) fusion proteins in *E. coli*. Such GST-kinase fusion proteins can be bound to glutathione-agarose beads to create purification columns with different protein kinases as baits. Other alternative methods for making kinase-columns for affinity purification of associating proteins also exist. For another example, the bait kinase could be fused to avidin and bound to biotin-linked agarose beads. The bait kinase could also be directly linked by cyanogen bromide to agarose beads. Crude cellular extracts in which the bait kinase is highly expressed would be expected to also contain physiologically interacting proteins that are effectors, regulators and substrates.

From Tables 7 and 8 for example, Kinase A lies upstream of Kinase B, and Kinase B lie upstream of Kinase C in order of their phosphorylation. However, it is unclear if these protein kinases directly interact. If they do, then immunoprecipitates of a cell extract that contains all three of these kinases would demonstrate that:

1. with immunoprecipitating antibody for Kinase A, Kinase B is co-immunoprecipitated as revealed by Western blotting of the immunoprecipitate with an antibody for Kinase B;
2. with immunoprecipitating antibody for Kinase B, Kinase A and C are co-immunoprecipitated as revealed by Western blotting of the immunoprecipitate with antibodies for Kinases A and C;
3. with immunoprecipitating antibody for Kinase C, Kinase B is co-immunoprecipitated as revealed by Western blotting of the immunoprecipitate with an antibody for Kinase B.

GST-fusion proteins for Kinases A, B and C, may be used as affinity ligands to purify interacting proteins from crude cellular extracts, then immunoblotting of the proteins that are retained by glutathione-agarose beads with the bound GST-kinases should demonstrate that:

1. with GST-Kinase A, Kinase B is purified as revealed by Western blotting of the agarose beads with an antibody for Kinase B;

2. with GST-Kinase B, Kinase A and C are purified as revealed by Western blotting of the agarose beads with antibodies for Kinases A and C;
3. with GST-Kinase C, Kinase B is purified as revealed by Western blotting of the agarose beads with an antibody for Kinase B.

It is important in such GST-kinase affinity purification experiments that proper controls are adopted to ensure that the interactions of kinases detected by this method are specific. For example, the binding of kinases to GST alone must be examined. With a wide range of protein kinase antibodies available for detection, the prospects of uncovering previously unpredicted kinase interactions improves. Moreover, it becomes possible to compare the magnitude of the signal for interaction with different kinases to focus on those that appear to be the most intense and likely the most important.

· *Alternative strategies for uncovering protein kinase interactions*

There are other techniques that may be used to establish possible protein-protein interactions. Some of these are based on the construction of fusion proteins that upon interaction assemble a functional transcription factor linked to a gene reporter system or permit a fluorescent reaction. The most widely used example of this approach is the yeast dihybrid system. In this method, a kinase is produced as a fusion protein with, for example, the DNA binding domain of a transcription factor such as Gal4 or LexA. A library of yeast that contain a diversity of proteins fused to the transactivation domain of the same transcription factor is transfected with the bait kinase construct. Those yeast that also produce a fusion protein that can interact with the bait kinase now have a functional transcription factor that can turn on the expression of a reporter gene (for example, β-galactosidase), which can produce a colour change in the yeast. These yeast can be isolated and the nucleotide sequence of the fusion protein that interacted with the bait kinase can be determined for the identification of the protein.

In another approach, an expression library of bacteria, yeast or other cells could be probed with a radioactively labelled protein kinase. The kinase could be radio-labelled with [^{32}P]phosphate following incubation with [γ-^{32}P]ATP by autophosphorylation or with ^{125}I. The probing would be performed on a nitrocellulose membrane containing the lysates from a replica plate of the expression library. Those colonies of cells that express a protein that interacts with the radiolabelled kinase can be isolated and the nucleotide sequence of the expressed proteins deduced for its identification.

In a variation of this method, the radiolabelled protein kinase could be used to probe a nitrocellulose blot of a cell or tissue extracts following SDS-polyacrylamide gel electrophoresis. In this case, the protein band that binds the radioactive probe, as visualized with a phosphoImager or autoradiography, can be subjected to protein micriosequencing for its identification.

A disadvantage of many of the protein-protein interaction identification techniques that are DNA-based is that many spurious and non-physiological complexes can be detected. However, in combination with the multi-kinase immunoblotting analysis, it is possible to confirm these interactions in physiological systems.

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Figure 1. **Comparison of the multi-kinase immunoblotting patterns of different rat tissues.** Electrophoresis of detergent solubilized lysates prepared from rat brain, heart and skeletal muscle was performed, and the positions of various protein kinases was visualized by ECL detection. Protein kinases of smaller size migrated correspondingly closer to the bottom of the SDS-PAGE gel. Each of the 18 strips derived from each SDS-PAGE gel were probed with different panels of protein kinase antibodies.

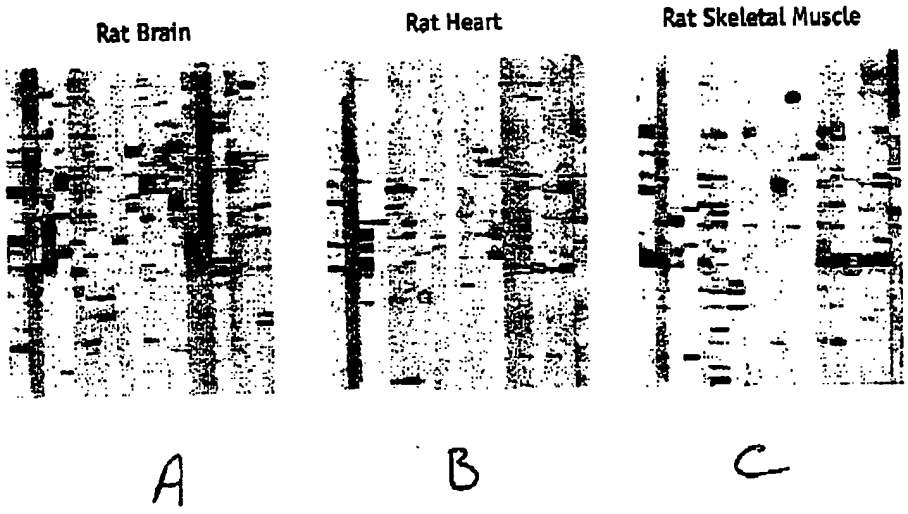


Figure 2. **Effects of anti-IgM treatment for 5 min on protein kinases in the human Ramos B cell line.** Electrophoresis of detergent solubilized lysates prepared from Ramos cells untreated (-) or exposed (+) to anti-IgM polyclonal antibody for 5 min was performed in alternating lanes, and the positions of various protein kinases was visualized by ECL detection. Each of the 14 paired strips derived from two SDS-PAGE gels were probed with different panels of protein kinase antibodies.

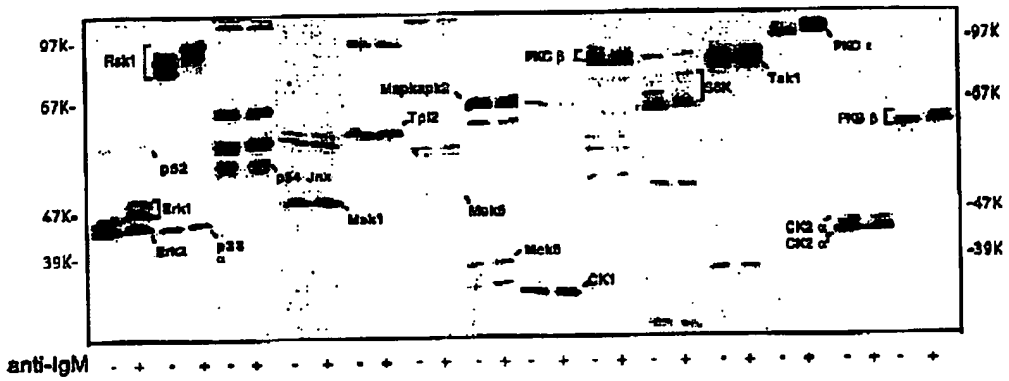
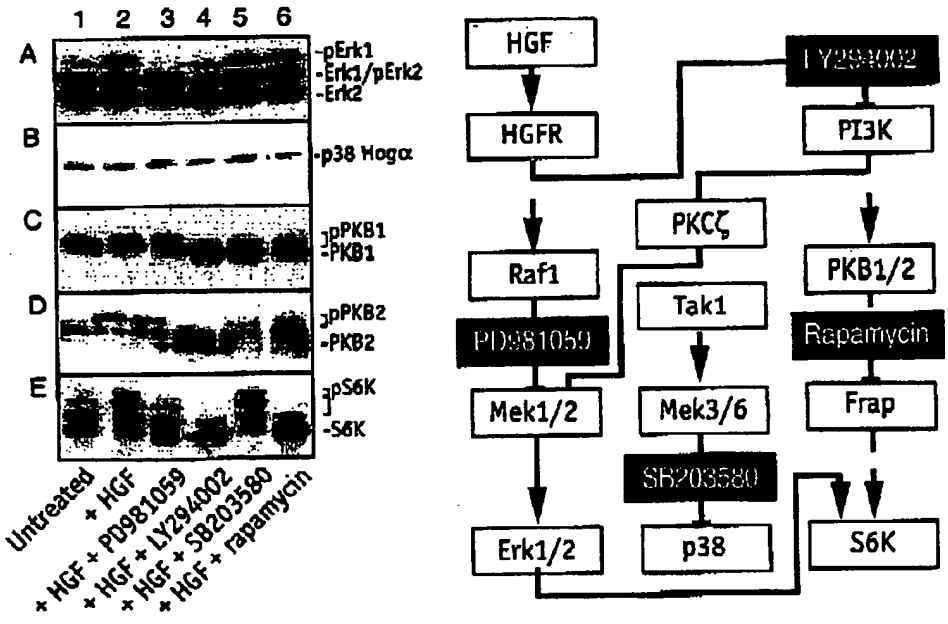
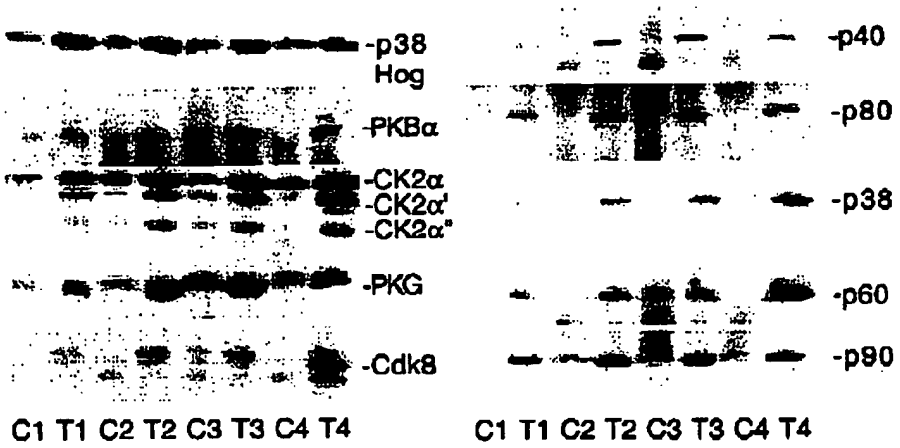


Figure 3. **Differential effects of kinase inhibitors on band shifting of selected protein kinases.** Electrophoresis of detergent solubilized lysates prepared from human ovarian surface epithelial cells were untreated (Lane 1) or exposed to 20 ng/ml of human hepatocyte growth factor (HGF) in the absence (Lane 2) or presence of PD98059 (Lane 3), SB203580 (Lane 4), LY294002 (Lane 5) or rapamycin (Lane 6) was performed. The effects of these treatments on the positions of Erk1 and Erk2 (Panel A), p38 Hog MAP kinase (Panel B), PKB1 (Panel C), PKB2 (Panel D) and S6 kinase (Panel E) as visualized by ECL detection are shown. The phosphorylated and band-shifted forms of these kinases are denoted with a "p" before their name. The partial structures of the protein kinases pathways in which these enzymes operate and the known sites of action of these drugs are shown on the right.



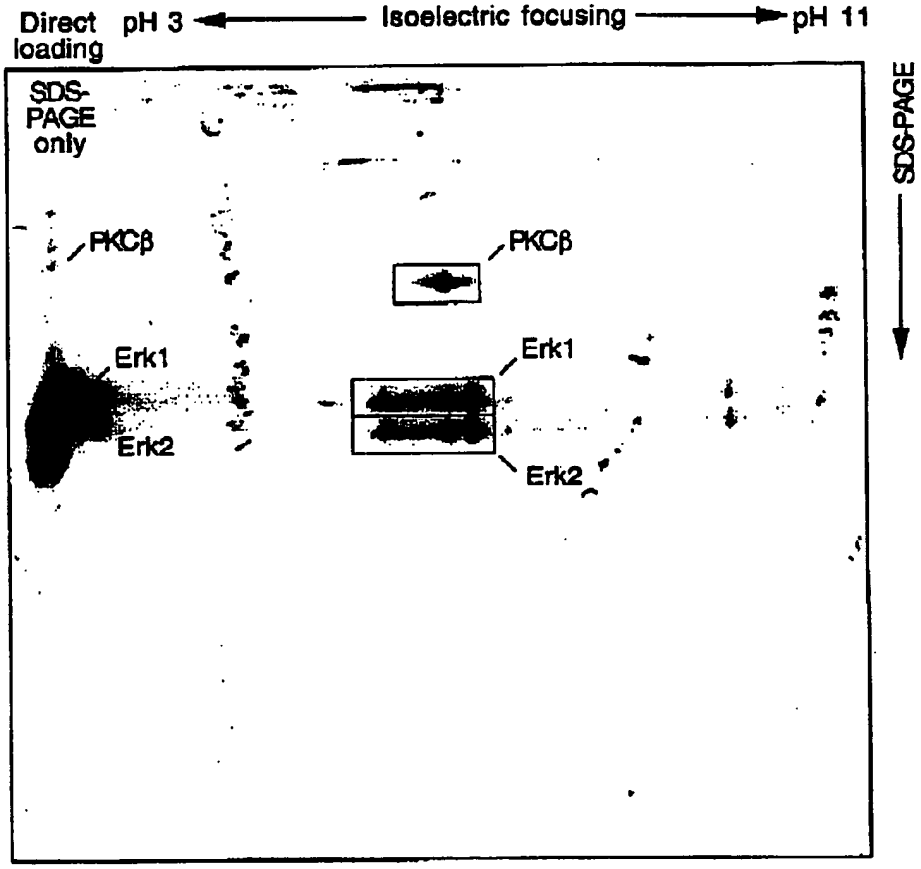
A B

Figure 4. Detection of known kinases and putative kinases in normal and tumor breast tumour biopsy samples of four human patients. Detergent solubilized lysates prepared from tumour (T) and adjacent control (C) breast tissue were subjected to multi-kinase profiling. In the left panels, the increased levels of p38 MAP kinase, protein kinase B-α (PKBα), casein kinase 2 (CK2), protein kinase G (PKG) and cyclin-dependent kinase 8 (Cdk8) in the tumor samples is evident. Five of 12 proteins that were demonstrated to be elevated in tumours and not yet known for their identity are shown on the right in Figure 4.



A B

Figure 5. Separation of Erk1, Erk2 and protein kinase C-β by 2D gel electrophoresis. Detergent solubilized rat brain extract (1 mg protein) was subjected to isoelectric focusing and SDS-PAGE. In the left most lane, 200 µg of the brain extract was directly applied to the same SDS-PAGE gel. Following 2D gel electrophoresis, the proteins were transferred to a nitrocellulose membrane, which was probed with antibodies for Erk1, Erk2 and PKC-β. The resulting Western blot is shown.



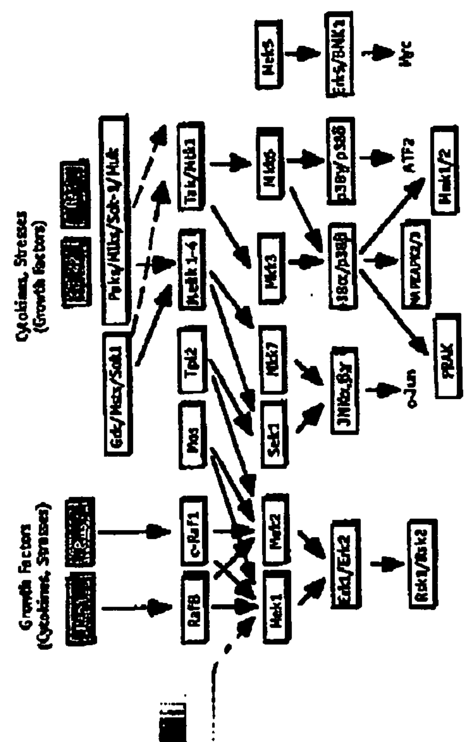
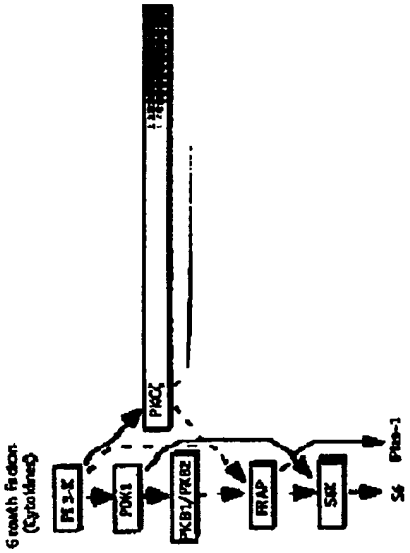

Growth Factors
(Cytokines, Stresses)
Cytokines, Stresses
(Growth factors)
RafB
c-Raf1
Mos
Tpl2
Gck/Mess/Sok1
Mek1
Mek2
Sek1
Mkk7
Mkk3
Mkk6
Mek5
Erk1/Erk2
Rsk1/Rsk2
c-Jun
ATF2
PRAK
Mnk1/2
Myc

FIG. 6


PI3-K
PDK1
PKB/PKB2
IRAP
SGK